ANALYSIS OF PATHOGENESIS IN THE EYESPOT FUNGUS, *PSEUDOCERCOSPORELLA HERPOTRICHOIDES*

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ABSTRACT

Field strains, W x R hybrids and inter-specific hybrids of the eyespot pathogen *Pseudocercosporella herpotrichoides* (Fron) Deighton were characterised using traditional morphological and more recently developed molecular identification techniques. This distinguished between the W and R pathotypes. The pathogenicity of these strains and hybrids to wheat, barley and rye was determined and related to their morphological and molecular characterisation. This highlighted the unreliability of some of these techniques. Characterisation of the hybrids revealed that the genetic inheritance from the parental strains was generally greater from the R-type parent but some W x R- hybrids showed an intermediate morphology and others showed novel pathogenicity.

An examination of the ability of the strains and hybrids to produce infection plaques *in vitro* found that infection plaque production could be related to the strain/hybrid pathogenicity on wheat, barley and rye. This indicates a requirement of infection plaques for successful host colonisation. No evidence was found of secondary metabolites produced in fungal culture filtrates using a wheat cell suspension assay nor in a wheat root inhibition assay which could be involved in pathogenicity or host symptom induction.

In a comparative study over time on wheat and rye, the development of disease symptoms was related to microscopic infection structures and the amount of fungal DNA present in the stem base. This revealed different infection strategies for the W and R pathotypes, the W-types being 'slow and steady' and the R-types being 'fast and furious' in their infection. The infection structures of both W and R-types were found to be always in advance of disease symptoms and limited colonisation of the host could occur without disease symptoms being present.

Colour mutants were produced from darkly pigmented W-type strains and tests were performed on their pathogenicity to wheat, their ability to survive UV irradiation and desiccation and oxidation. An analysis was made of their structure using transmission electron microscopy. The results of these experiments revealed that the primary role of melanin in this fungus is in environmental protection. The use of melanin biosynthetic inhibiting compounds indicated that melanin biosynthesis in this fungus probably occurs via the 1,8-dihydroxynaphthalene (DHN) pathway. A yellow pigment was detected and is suggested to be a carotenoid. Genetic analysis of the colour mutants revealed that a possible single unlinked gene was causing the albino phenotype.

DEDICATED TO MY PARENTS

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LIST OF ABBREVIATIONS

1,3,6,8-THN	1,3,6,8-tetrahydroxynaphthalene
1,3,8-THN	1,3,8-trihydroxynaphthalene
2-HJ	2-hydroxyjuglone
ADAS	Agricultural Development and Advisory Service
ANOVA	Analysis of variance
ANS	8-anilino-sulphonic-acid
CTAB	Hexadecyl trimethylammonium bromide
dATP	Adenine tri-phosphate
dCTP	Cytosine tri-phosphate
dGTP	Guanine tri-phosphate
DHN	1,8-dihydroxynaphthalene
DNA	Deoxyribonucleic acid
DOPA	3,4-dihydroxyphenylalanine
dTTP	Thamine tri-phosphate
ELISA	Enzyme linked immunosorbent assay
F/E	Fast/Even
GBHD	γ-glutaminyl-3,4-dihydroxybenzene
GUS	β-glucuronidase
ITS	Internal transcribed spacer
mtDNA	Mitochondrial DNA
MYG	Malt, yeast glucose
NADPH	Reduced nicotinamide adenine dinucleotide phosphate
nDNA	Nuclear DNA
Pc	Plastocyanin
PCR	Polymerase chain reaction
PG	Polygalacturonase
PL	Pectin lyase
PME	Pectin methyl esterase
RAPD	Random amplified polymorphic DNA

rDNA	Ribosomal DNA
RFLP	Restriction fragment length polymorphisms
RNA	Ribonucleic acid
rpm	Revolutions per minute
rRNA	Ribosomal ribonucleic acid
S/F	Slow/Feathery
SDW	Sterile distilled water
SOD	Superoxide dismutase
TAE	Tris-acetate EDTA
TE	Tris EDTA
TWA	Tap water agar

CHAPTER 1

INTRODUCTION AND RATIONALE

The fungus *Pseudocercosporella herpotrichoides* (Fron) Deighton (anamorph) causes eyespot, a stem base disease of cereal crops in temperate climates. It was first discovered at the beginning of the 20th century in France and the USA and then in the UK in 1935 by Glynne. Following the development of fungicide resistance during the 1980's and changes in agricultural practices including earlier sowing dates, it has increasingly become an important pathogen in many countries including the UK (Fitt, Goulds and Polley, 1988). Initial research concentrated on the fungus and its associated disease symptoms, yield loss and its epidemiology (Scott, 1971; Scott and Hollins, 1974). Pathotype identification was important and became more so when population changes were found to be occurring in the field (King and Griffin, 1985). The first method of pathotype differentiation was based on seedling pathogenicity which then became combined with cultural morphology *in vitro* (Lange de la Camp, 1966; Hollins, Scott and Paine, 1985). Molecular techniques are now used. During the last decade the discovery of the sexual stage with the teleomorphs *Tapesia yallundae* Wallwork & Spooner and *T. acuformis* (Boerema, Pieters & Hamers) Crous corresponding to W and R-pathotypes respectively has added a new dimension to this disease (Nicholson *et al.,* 1995; Dyer *et al.,* 1996). Understanding this sexual stage in connection with the pathotypes is now important in increasing our knowledge of this fungus, its interaction with the host plants and ensuring its efficient control.

Continued research into this disease is necessary because pathogen populations have become resistant to the fungicides used to control them and earlier sowing dates have favoured the development of severe epidemics. The current indication that the W and R-pathotypes are separate species verifies a need for expansion on the comparative biology of these types. The epidemiology of the W and R -types has also been found to be different with the later development of infections by the R-types compared to the W-types (Goulds and Fitt, 1990). The increase in our understanding of the

relationship of the pathotypes with the host plants may have improved the accuracy in predicting the severity of eyespot epidemics. This might prevent unnecessary spraying and severe late epidemics. There is also a need for new sources of resistance to be identified and incorporated into new cultivars and new targets are required for fungicide control.

The relatively recent development of DNA methods for pathotype identification and quantification directly from host stem bases now allows the relationship between fungal 'biomass' and symptom expression and host colonisation to be explored directly. The availability of W x R recombinant hybrids with various levels of pathogenic ability provides an opportunity to use these techniques to explore which factors are important for pathogenic behaviour.

The present study used the new DNA methods and W x R hybrids with the aim of expanding the known biology of the main W and R-types of this fungus by examining their relationships with the cereal hosts, and exploring mechanisms which might be involved in pathogenicity. The primary work compared the traditional morphological and pathogenicity identification techniques with the newer molecular identification techniques on field isolated strains and parasexual recombinant hybrids (W x R and inter-specific). The role of infection plaques and secondary metabolites in pathogenicity was then investigated *in vitro* and related to the pathogenicity results. The second part of this study examined a time course of infection using selected W and R- types and hybrids by examining the relationship between the disease symptoms seen on the host and the presence of microscopic infection structures. This relationship was then compared to the quantity of fungal DNA present in the plant stem base. The final part of this work examined the role of melanin in this fungus, its biosynthetic pathway and possible genes involved.

CHAPTER 2

LITERATURE REVIEW

2.1 Eyespot

Eyespot, caused by the ascomycete *P. herpotrichoides*, (teleomorphs: *T. yallundae* and *T. acuformis* (Dyer *et al.*,1996; Nicholson *et al.*, 1995)), is an economically important disease of cereals in temperate regions of the world, occurring in Europe, the USSR, South Africa, parts of North America and Australasia (Fitt *et al.*, 1988). In the UK the disease is most severe on autumn-sown wheat (*Triticum aestivum* L.) and barley (*Hordeum vulgare* L.) crops but it can also affect rye (*Secale cereale* L.), oats (*Avena sativa* L.), triticale, and spring-sown crops (Fitt, 1988). It is also an important component of the parasitic complex of cereal stem base rot in association with *Rhizoctonia* spp. DC., *Microdochium nivale* (Fries) Samuels & Hallet varieties and *Fusarium* spp. Link (Goulds and Polley, 1990).

2.1.1 Disease symptoms

Eyespot takes its name from the disease symptoms, which are elliptical shaped lesions typically appearing at the stem base just above the soil. The lesions have a creamy centre and a black necrotic margin (Plate 2.1). Initially the lesions are diffuse with a brown appearance but as the infection progresses, the fungus spreads in the outer leaf sheaths and then penetrates the inner ones and the lesions become more defined (Fitt, 1988). When infected straws are split open the lumen of the hollow stem in the region of the lesion may be filled with a grey fungal mycelial growth. If lesions become severe they may cause the stem to become rigid, kinked, frayed or otherwise distorted and weakened. The straw may bend or break at this point (Fitt, 1988). The weakening of the stems may cause them to lodge before harvest in many directions or alternatively they may be lodged and fall in one direction by stormy weather. Mature crops infected later in the season or in which infection progress is less rapid may produce ears that die prematurely, have a reduced number and shrivelled grain per ear. In addition the ears may become discoloured producing a

symptom known as 'whiteheads'. These ears may be then colonised by secondary moulds and turn black especially in wet weather (Scott and Hollins, 1974; Fitt, 1988, 1992).

2.1.2 Economic losses caused by eyespot

Economic losses caused by eyespot are attributed to the effect of the lesions which girdle and soften the stem base, interfering with sap circulation. Indirect losses are caused by lodging which can result in the financial loss being probably greater than the direct effect on yield, since the quality of grain from lodged crops is low. Eyespot reduces yields of winter crops only when there is a high incidence of moderate or severe lesions on stems with losses unlikely to exceed 50% even with all shoots severely infected (Scott and Hollins, 1974; Clarkson, 1981). Yield losses of around 40% have been measured in pot and field experiments and related to the incidence of severe eyespot lesions at the time of harvest (Scott and Hollins, 1974; Clarkson, 1981; Davis and Gareth Jones, 1970). The importance of eyespot epidemics has increased over the years with estimated losses in winter wheat and barley crops at £19 million in 1989 despite fungicidal control measures estimated at £17 million in that year (Fitt, 1988, Fitt *et al.,* 1990).

2.1.3 Pathotypes

A hyphomycete fungal pathogen associated with the disease symptoms was first described as *Cercosporella herpotrichoides* by Fron in 1912 and was later re-named as *P. herpotrichoides* (Fron) Deighton (Deighton, 1973). Field populations of the fungus comprise of two main pathotypes, the wheat type (W-type) and rye type (R-type) which were originally distinguished on the basis of seedling pathogenicity tests. The W-types are highly pathogenic on wheat but are only slightly pathogenic on rye and certain grasses, whereas R-types are generally equally pathogenic on wheat and rye (Lange de la Camp, 1966; Scott, Hollins and Muir, 1975; Scott and Hollins, 1980). There is however considerable overlap between W and R-types on both hosts (Mauler and Fehrmann, 1987b, Creighton, Cavelier and Fitt, 1989). Morphological

growth studies on agar distinguished the two pathotypes further with the W-type producing green to grey colonies with smooth margins and having a high growth rate (F/E) and the R-type producing beige to brown colonies with feathery margins, but having a growth rate at approximately half the rate of W-type isolates (S/F) (Plate 2.1) (Hollins *et al.,* 1985; King and Griffin, 1985; Sanders, de Waard and Loerakkar, 1986). Later studies found that R-type isolates are able to sector whilst in culture to produce variants with a morphology resembling W-type colonies (Hocart, 1987; Julian, Hardy and Lucas, 1994). Isolates have also been differentiated by the colour of pigment produced in culture when grown on maize meal agar under near UV light (Creighton, 1989). However, it has been found that pathogenicity has not always correlated with cultural characteristics (Nicholson, Rezanoor and Hollins, 1993; Creighton, 1989).

Nirenberg (1981) divided the German isolates of the fungus into two varieties based on spore morphology and size. *P. herpotrichoides* var. *herpotrichoides* Nirenberg conidia were 35-80μm and either straight or curved, *P. herpotrichoides* var. *acuformis* Nirenberg conidia were 43-120μm and generally straight. It has been suggested that these varieties might correspond to the W and R-types respectively (King and Griffin, 1985; Sanders *et al.,* 1986). Fitt, Creighton and Bateman (1987). However found little difference between conidia of W and R-types except conidia form the W-type isolates were slightly more curved.

W and R-type isolates have been distinguished on the basis of isozyme polymorphisms, with isozyme patterns within pathotypes being similar for isolates of diverse geographical origins (Julian and Lucas, 1990; Priestly *et al.,* 1992). The W and R-types have also been differentiated on the basis of polymorphisms of nuclear DNA (Thomas, Maraite and Boutry, 1992; Takeuchi and Kuninaga, 1994), mitochondrial DNA (Nicholson *et al.,* 1993; Takeuchi and Kuninaga, 1996) and random amplified polymorphic DNA (RAPD) markers (Nicholson and Rezanoor, 1994). Ribosomal internal transcribed spacer (ITS) region sequences (Poupard *et al.,*

1993; Gac, 1991, Gac, Montfort and Cavelier, 1996), restriction fragment length polymorphisms (RFLP) of total DNA and ribosomal DNA (Nicholson *et al.,* 1991b) have also been used. Most recently, specific primers have been generated from RAPDs for use in a polymerase chain reaction (PCR) for detection of W and R-types in plant tissues (Nicholson *et al.,* 1997). These results have indicated major differences between the genomes of the W and R-types.

In addition to the W and R-types, two other pathotypes are known, S-type isolates which are pathogenic on wheat and *Aegilops squarrosa* L. (Scott *et al.,* 1976; Scott and Hollins, 1980) and C-types which are pathogenic on wheat, *A. squarrosa* and *Agropyron repens* L. (Cunningham, 1965, 1981). In culture the C-type morphology has been found to be indistinguishable from a W-type (Nicholson *et al.,* 1991b) and has a similar mitochondrial DNA and RFLP profile (Nicholson *et al.,* 1993). Isozyme profiles of C-types indicate that they might represent a genetically distinct subgroup within the W-type group (Priestly *et al.,* 1992).

2.1.4 Sexual cycle

The teleomorph of *P. herpotrichoides* was first identified in Australia when apothecia were found to develop on infected straw stubble *in vitro* (Wallwork, 1987). Single ascospores of the newly described species *T. yallundae* germinated to form characteristic colonies and conidia of the W-type of *P. herpotrichoides* (Wallwork and Spooner, 1988). Later studies found that infection of wheat seedlings by ascospores of *T. yallundae* was similar to infection from rain-splash-dispersed W-type conidia (Daniels *et al.,* 1995). Apothecia were subsequently found in New Zealand (Sanderson and King, 1988), Germany (King, 1990, 1991), Belgium (Moreau, Van Schingen and Maraite, 1989) and England (Hunter, 1989; Dyer *et al.,* 1994a; Dyer and Lucas, 1995). Conditions for apothecia development in the field have been found to be associated with low monthly temperatures (3-8°C) (Dyer *et al.,* 1994b). The development of an *in vitro* crossing protocol revealed that *T. yallundae* exhibits two allele heterothallism, with the two mating types designated

MAT1-1 and MAT1-2 (Nicholson, Rezanoor and Hollins, 1991a; Dyer *et al.,* 1993b). In contrast, there have been fewer reports of R-type progeny being obtained from apothecia, but they have been found on straw stubble in Germany (King, 1990) and England (Dyer *et al.,* 1994b). They also display a heterothallic mating system (Dyer *et al.,* 1996). The reason for the rarity of sexual reproduction in the R-type is unclear, but this may be due to a lack of compatible isolates in the field or of a particular environmental trigger required to instigate sexual reproduction. Further studies of *in vitro* crosses have found that W-type isolates are able to sexually mate with C and S-types but not R-types, thus the three pathotypes (W, C and S) form a single mating population. The W and R-types appear to represent different biological species based upon the failure of isolates from the two groups to inter-cross (Nicholson *et al.,* 1995; Dyer *et al.,* 1996). It has been proposed that the two groups WCS- and R-type be referred to as *T. yallundae* and *T. acuformis* respectively (Robbertse, Campbell and Crous, 1995; Nicholson *et al.,* 1997). The occurrence of the sexual cycle is of importance as it can lead to greater variation within the eyespot population due to recombination and segregation.

2.1.5 Parasexual cycle

The discovery of the teleomorph of *P. herpotrichoides* allows for genetic analysis of this pathogen through the ordered segregation of the parental genomes. However where mating type incompatibility or sexual infertility limits the number or range of crosses, the parasexual system may be more successful. The parasexual cycle allows for genetic re-assortment to take place via the fusion of two nuclei within a heterokaryon giving rise to a somatic diploid nucleus which may revert to the haploid state as chromosomes are lost by non-disjunction during subsequent mitotic divisions. The cycle is found naturally in some fungi and in many species it can be induced in the laboratory. It was first described in *Aspergillus nidulans* (Eidam) G. Wint (Pontecorvo *et al.,* 1953) and was demonstrated in *P. herpotrichoides* by Hocart, Lucas and Peberdy (1993b). Hocart *et al,* (1993b) used complementary recessive genetic characters, typically auxotrophic, assimilation or pigmentation

mutants to detect compatible hyphal fusion events. The cycle followed a typical pattern, consisting of a mosaic heterokaryon phase which allows for the mixing of the two cytoplasms and organelle exchange, followed by nuclear fusion giving a relatively stable diploid phase, and then various putative aneuploid stages leading to recombinant haploid formation. Stages in the parasexual cycle between W and R-types differed from that seen in the original intrastrain crosses involving fully vegetatively compatible strains (Hocart, Lucas and Peberdy, 1993a) because of vegetative incompatibility found to exist between the W and R-types (Hocart, Lucas and Peberdy, 1989). The fusion of protoplasts removed this vegetative incompatibility which appeared to be expressed primarily at the level of the cell wall rather than at an intracellular level (Hocart *et al.,* 1993a). It was found that in contrast to vegetative compatible strains no heterokaryon phase could be detected. It was suggested that this could be due to the mosaic structure of the heterokaryon, which requires repeated hyphal anastomosis between complementary partners to form. Fusion products were considered to represent diploid colonies with stable progeny recovered after either spontaneous breakdown or exposure of the fusion products to haploidising agents such as carbendazim. Recombinant progeny occurred either by mitotic recombination where crossing over occurred between non-sister chromatids during mitosis, or following non-disjunction within the haploid nucleus during mitosis. The hybrid progeny obtained showed segregation of morphology and pathogenic abilities.

Another species of *Pseudocercosporella* infecting cereals has been identified and named *P. anguioides* Nirenberg. It is found throughout Germany and has been reported in the UK (Nirenberg, 1981; Bateman, 1988). It is associated with cereal stem bases but is not considered economically important as it is only weakly pathogenic. However its presence may be a possible source for confusion when assessing crops for the presence of eyespot disease (Bateman, 1988). Inter-specific hybridisation between both W and R-types of *P. herpotrichoides* and *P. anguioides* via protoplast fusion indicated the similarity of genome organisation and that these

species are relatively closely related (Hocart and McNaughton, 1994).

2.1.6 Epidemiology

Eyespot is favoured by mild, wet winter weather and cool damp spring weather. It is most severe in early sown autumn wheat and barley crops. Spring crops in the drier eastern areas of England are rarely badly affected but in certain western areas of England, Scotland, Wales and Ireland wet winter and spring weather usually leads to eyespot being a problem on spring sown crops. Eyespot is most severe on land growing continuous wheat or barley crops and under these intensive regiemes eyespot epidemics become severe within a few years. The first cereal crop after several years of grass usually has little or no eyespot but breaks of less than 2 years are generally ineffective in controlling the disease (Cox and Cock, 1962; Fitt, 1988).

In the absence of a suitable host plant *P. herpotrichoides* survives for about 2-3 years as a saprophyte on host residues (Macer, 1961a, 1961b) although it has a low competitive saprophytic ability. Crop succession is therefore fundamentally important for disease build up. Infected straw on the soil surface is the main source of inoculum for development of new epidemics although self sown wheat and barley, and grass species may also be a source. Successful infection requires the presence of infectious crop residues close to the soil surface which is influenced by crop succession and soil tillage. Soil tillage by ploughing usually buries host residues from a past host crop but it can however carry some residues back to the surface increasing primary infection risk if a host crop is going to be sown (Colbach and Meynard, 1995). Buried straw retains the ability to produce spores longer than straw left on the soil surface (Macer, 1961b). In the UK sporulation occurs on the infected straw throughout the winter but declines rapidly in the spring although sporulation can continue to July (Hollins and Scott, 1980; Fitt and Bainbridge, 1983).

The life cycle of *P. herpotrichoides* on wheat from initial infection by conidia to lesion development can be seen in Fig 2.1 taken from Fitt *et al*, (1988). Sporulation

can occur at temperatures between 1-20°C with the optimum temperature on wheat straw being close to 5°C (Jorgensen, 1964). A higher optimum temperature for sporulation (10-15°C) has been observed in pure culture (Rowe and Powelson, 1973a), but on naturally infected debris this range of temperature would stimulate the growth of competing fungi. Temperatures in the range 20-25°C stimulate the multiplication of bacteria (Higgins and Fitt, 1984). Experiments monitoring fluctuating temperatures have resulted in a value, the "Daily Thermal Sporulation Coefficient" which identified periods from field temperatures when sporulation might occur, thus assessing the seasonal epidemic potential (Rowe and Powelson, 1973a). Water has been suggested to be essential for sporulation on straw (Glynne, 1953; Rowe and Powelson, 1973a) but there has been conflicting evidence on the role of light. Conidia of *P. herpotrichoides* can be splashed dispersed over a short distance; evidence for this has been obtained using simulated raindrops falling on spore suspension and infected debris using photographic film (Fatemi and Fitt, 1983; Fitt and Lysandrou, 1984; Fitt and Nijman, 1983; Huber, Fitt and McCartney, 1996). Field site experiments using photographic film found that no spores were collected in the first few minutes of rainfall suggesting that the mucilage in which they were produced must be dissolved before they are released into a spore suspension and become available for dispersal (Fitt and Bainbridge, 1983). In still air, most spores are deposited within 1m of the source and even with increasing wind-speeds few spores travel beyond 2m (Fitt and Nijman, 1983). Some conidia become airborne and might be carried considerable distances by the wind but how long they remain viable or whether they reach sufficient numbers to cause a lesion may limit their role in the epidemiology of eyespot. It has been found that lesions developed more rapidly from larger inoculum doses but they could also be initiated by a few spores (Higgins and Fitt, 1984).

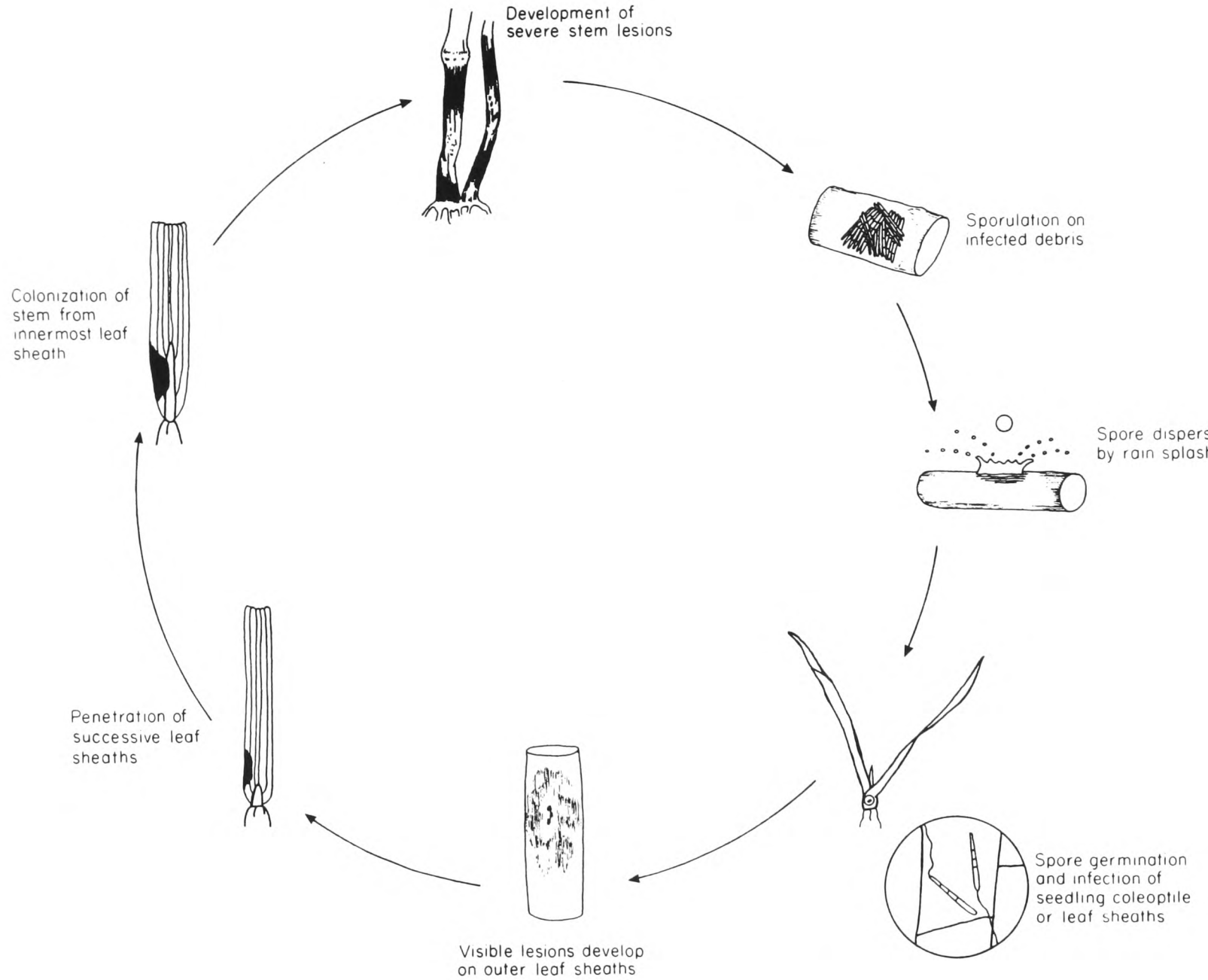


Fig 2.1: Stages in the development of eyespot lesions in winter wheat crops (Fitt *et al.,* 1988)

Ejected ascospores from apothecia on straw stubble probably provide a source of airborne inoculum for long range dispersal of the disease (Sanderson and King, 1988). However, the importance of the sexual stage to the epidemiology of the pathogen has remained uncertain, since little is known about the formation of apothecia in the field (Fitt, 1992) and the dispersal and infection of the ascospores to cereal crops throughout the growing season. The occurrence of apothecia in England and in other European countries may have increased because of the current policy of "set-aside" (Bateman, Dyer and Manzhula, 1995). However for apothecia to be produced, "set-aside" fields after cereals need to be left uncultivated, with standing straw stubble, through the main period of their production. This is approximately 5 to 7 months after harvest with temperatures between 3°C and 8°C (Dyer *et al.*, 1994b).

Infection by *P. herpotrichoides* has been shown to occur at temperatures between 6-18°C (Lange-de la Camp, 1966, 1967; Scott, 1971; Bateman and Taylor, 1976a, 1976b; Higgins and Fitt, 1985a) but the optimum and range of conditions is still to be precisely established. The penetration of the stem by the W-type occurs earlier than that by the R-type which increases in its rate of re-isolation in relation to the W-type as the growing season progresses (King and Griffin, 1985; Coskun, Bateman and Hollomon, 1987). Similarly the R-type has been shown to cause infections later in field inoculated plots (Goulds and Fitt, 1990). Progress of the pathotypes has been tracked by re-isolation of the fungus from the hosts and thus the differences found between the pathotypes throughout the growing season may reflect the ease at which the pathotypes can be re-isolated and perhaps not differences in epidemiology. Both types can exist in the same plant or even the same lesion and interaction between the pathotypes can influence the development of eyespot (Poupard and Cavelier, 1992). Stems weakened or altered by *P. herpotrichoides* may be a suitable substrate for a secondary coloniser such as *Fusarium* spp., which may begin infection at a distance from the original lesion. It may not cause distinct symptoms by itself (Bateman, 1993). PCR primers specific to the main stem base complex species used directly on stem base samples now enable *P. herpotrichoides* pathotype identification without

the need for re-isolation. They can also can identify other colonisers present (Nicholson *et al.,* 1996, 1997).

2.1.7 Life cycle

The coleoptile is most susceptible to infection (Bateman and Taylor, 1976a, 1976b) although *P. herpotrichoides* can infect leaf sheaths later in the growing season when the coleoptiles have disintegrated (Hollins and Scott, 1980; Daniels, Lucas and Peberdy, 1991; Daniels *et al.,* 1995). Infection of the coleoptile occurs via appressoria produced by surface hyphae from the germinated conidia/ascospores (Bateman and Taylor, 1976a; Daniels *et al.,* 1991, 1995). Studies on the two pathotypes have revealed different patterns of coleoptile infection as is seen in Fig 2.2 taken from Daniels *et al,* (1991). The W-type spores germinate to produce superficial hyphae displaying orientated growth and form appressoria only in anticlinal cell wall junctions. The R-types grow more randomly and appressoria appear at a variety of positions. Mycelial plates composed of ramifying mycelium develop within and/ or on the coleoptile from which spores can be produced at the surface and within the interstices indicating the possible involvement of a secondary infection cycle. Runner hyphae are formed from these plates aid host colonisation. Leaf sheaths are then infected by infection plaques that develop between the coleoptile and the first leaf sheath. The W-type and R-type infection plaques differ in their morphology: the W-type plaques are asymmetrical and composed of loosely associated cells while the R-type plaques are discrete and circular and consist of closely associated cells. The inward progression of the pathogen then occurs from leaf sheath to leaf sheath with new infection plaques being formed on each successive leaf sheath until the pathogen reaches the hollow stem.

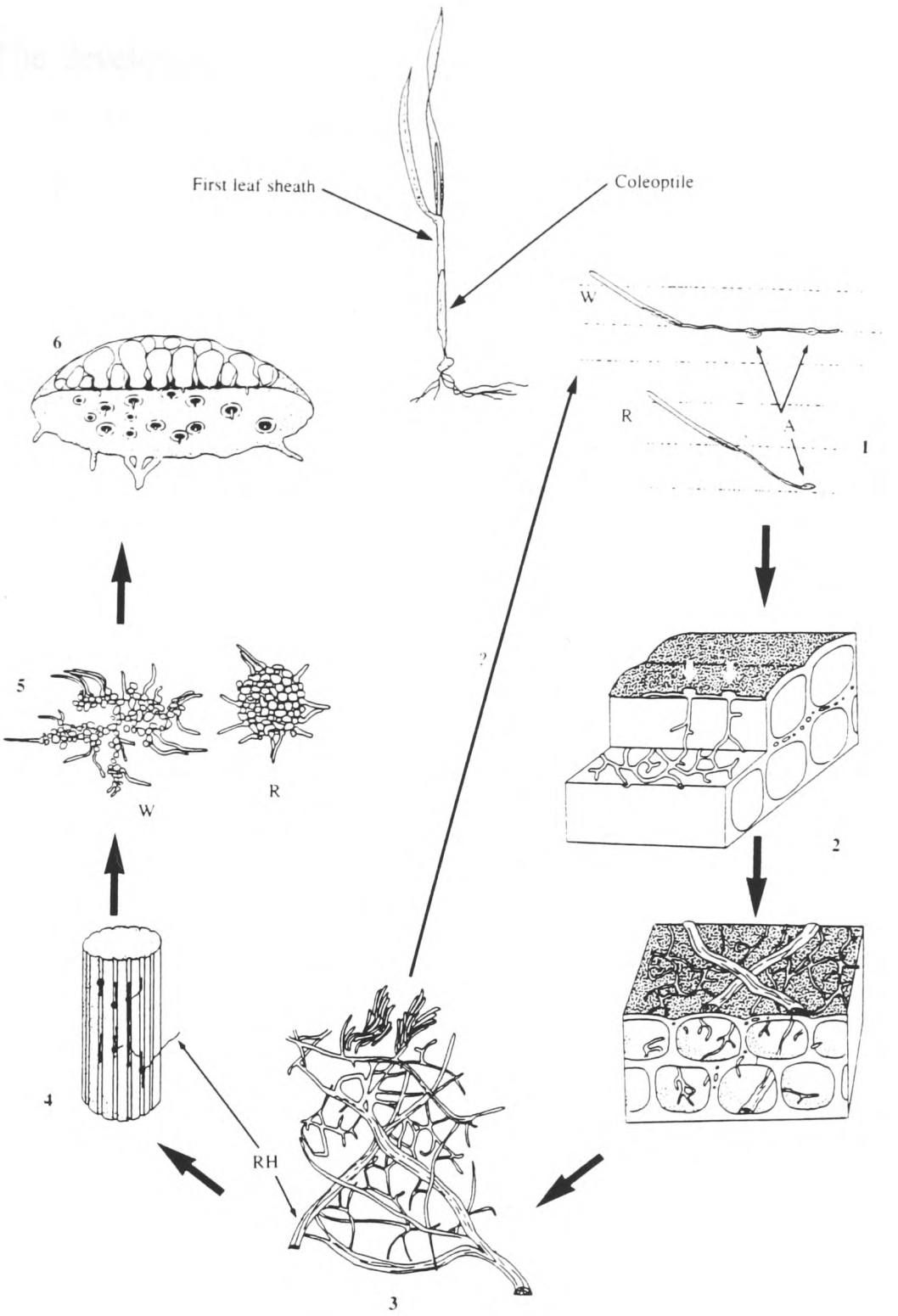


Fig 2.2: Infection stages of *P. herpotrichoides* W and R-types on the coleoptile and first leaf sheath of wheat seedlings (Daniels *et al.*, 1991).

1 Alignment or non-alignment of superficial hyphae showing appressorium (A) formation.
2 Coleoptile invasion and establishment of mycelial plates showing the intramural growth habitat of W-types (top) and the more random invasive pattern of R-types (below).
3 Secondary sporulation and formation of runner hyphae (RH) from mycelial plates.
4 Alignment of hyphae in vascular grooves and establishment of infection plaques.
5 Differentiation of infection plaques showing W-type and R-type plaque morphology.
6 Development of infection hyphae from the lower surface of the plaque.

After infection there is a period of several weeks before lesions are visible at the stem base. The development of lesions is influenced by the production of new inner leaf sheaths and the death and disintegration of the outer ones (Fitt *et al.*, 1988). Penetration of the leaf sheaths has been suggested to be a function of temperature because there is always sufficient water at the site of the lesion to support growth, with the number of leaf sheaths penetrated increasing with increasing temperature (Ponchet, 1959; Higgins and Fitt, 1985). However the rate of leaf sheath death also increases with increasing temperature thus alternatively restricting penetration through successive leaf sheaths (Higgins and Fitt, 1985a).

The incidence of eyespot is assessed as the proportion of plants or shoots with visible lesions, and from this it has been suggested that eyespot is a disease with one cycle per season (Rowe and Powelson, 1973b). This has been disputed as the theory assumes that the incidence of disease increases with time but it may decrease if lesions fail because the outer leaf sheaths die and decay before the fungus has colonised the ones below them (Fitt and White, 1988). Spores produced on the leaf sheaths are small by comparsion with the numbers produced on infested debris (Rowe and Powelson, 1973b). Thus most new shoots are infected by splash-dispersed conidia from infected debris (primary spread) or by mycelial growth from infected shoots on the same plants (secondary spread). It has been considered that secondary spread is the dominant mechanism for the infection of new shoots and the incidence of shoot infection can be fitted by an equation for polycyclic diseases (Rapidly *et al.*, 1979). To incorporate disease incidence and severity, a penetration index is used which is the number of leaf sheaths penetrated per infected plant multiplied by the proportion of infected plants (Higgins and Fitt, 1984).

2.1.8 Population changes

The fungicide sensitivity of both W and R-types of *P. herpotrichoides* changed during the 1980's from MBC (methyl benzimidazole-2-ylcarbamate, carbendazim) sensitive strains to MBC resistant strains as a consequence of the regular use of MBC

fungicides by growers. In the 1970's MBC fungicides (generating carbendazim, inhibiting β-microtubule assembly) were introduced to control eyespot and MBC resistance was first reported by Rashid and Schlosser in 1975. Initially the frequency of MBC resistant conidia detected was low and MBC fungicides continued to be used. However in 1981 MBC fungicides failed to control eyespot on some winter wheat crops and most isolates of the pathogen from the affected fields were found to be MBC resistant (Brown, Taylor and Epton, 1984). By the late 1980's the population of *P. herpotrichoides* on winter wheat and barley was found to be almost totally resistant to MBC fungicides (Yarham, 1986; Hollins and Scott, 1987). The incidence of MBC resistance isolates was found to increase rapidly when MBC fungicides were applied (Bateman *et al.,* 1985; Hoare, Hunter and Jordan, 1986).

In addition to a change in MBC fungicide sensitivity during the 1980s a population shift was seen with the original population composed of W-types being replaced by one consisting predominantly of R-types (Yarham, 1986; Hollins and Scott, 1987). The demethylation inhibiting (DMI) (inhibiting ergosterol biosynthesis) fungicides such as the imidazole prochloraz and several triazoles, including flusilazole, were introduced in the 1980's. It was found that the R-types were intrinsically resistant to the triazole derivatives compared to the W-types, but both types were sensitive to prochloraz. The use of these triazoles thus selected in favour of the R-type (Bateman, Goulds and Ainsley, 1990; Hoare *et al.,* 1986). The intensive use of these DMI fungicides in France led to the development of acquired resistance towards triazoles in the W-types and towards prochloraz in the R-types (Leroux and Gredt, 1997). The increase in the acreage of winter barley (King and Griffin , 1985) may also have selected in favour of R-type isolates.

The current use of the DMI fungicide prochloraz to control eyespot may also influence apothecial production and thus population changes. This is because prochloraz selects for the R-type (Leroux and Gredt, 1997) and sexual reproduction has been found only rarely in this type, occurring mainly between compatible strains

of the W-type fungus (Dyer *et al.,* 1993a). By controlling the disease, as well as selecting strains, the fungicides carbendazim and prochloraz therefore influence the amount of sexual reproduction (Bateman *et al.,* 1995). Application of prochloraz is likely to decrease the ability of the eyespot population to produce genetic variation where R-type resistance occurs. MBC fungicide resistant W-type strains in contrast are likely to produce apothecia and thus increase genetic variation (Bateman *et al.,* 1995).

2.1.9 Control of the disease

Control of eyespot in the UK relies on a combination of cultivar resistance, fungicides and cultural methods. The relative importance of each has changed over time according to socio-economic and political influences on agricultural practice. Cultivar resistance is a cheap method with breeding costs incorporated when growers buy the seed. Host resistance may take the form of direct resistance to the growth of the pathogen at the stem base by thickening of the hypodermis and secondary walls (Murray and Bruehl, 1983), or of indirect tolerance through resistance to lodging by having short stiff straws (Fitt *et al.,* 1990). Extensive replication is required to distinguish resistant and susceptibility to eyespot making studies of resistance complicated. Most UK cultivars of winter wheat incorporate resistance to eyespot derived from the French cultivar Cappelle-Desprez. The resistance is partial but disease severity and yield losses are generally less than susceptible cultivars (Hollins *et al.,* 1988). Capelle-Desprez resistance is coded for by one or more genes on chromosome 7A (Law *et al.,* 1975). Another wheat cultivar Rendezvous incorporates resistance from a wild grass *A. ventricosa* Tausch. The resistance is coded by a single dominant gene on chromosome 7D (Hollins *et al.,* 1988). This resistance gene was first transferred to a French breeding line VPM 1. The cultivar Rendezvous is more resistant than other winter wheat cultivars, including those with resistance from Cappelle-Desprez (Fitt *et al.,* 1990). Trials have indicated that the cultivar Rendezvous may contain resistance genes from Cappelle-Desprez and *A. ventricosa* as it is more resistant than its parent VPM 1 (Hollins *et al.,* 1988).

The introduction of MBC fungicides gave good yield increases for relatively small costs in comparison to the potential benefits and they were used routinely as 'insurance' against possible eyespot development. By 1981 the failure to control eyespot in two crops (King and Griffin, 1985) and subsequent Agricultural Development and Advisory Service (ADAS) surveys found that MBC resistance was widespread in the UK and by 1987 over 70% of isolates were MBC resistant (Fitt, 1988). An increase in the use of MBC fungicides has also reported in parts of Europe (Fitt *et al.,* 1988). It has been found that resistance to MBC fungicides results from inheritance of single resistance genes (Dyer and Lucas, 1995). The DMI fungicide prochloraz is now used either alone or in a mixture with MBC fungicides. It is less effective than MBC fungicides against MBC sensitive isolates (Hoare *et al.,* 1986) but it is effective against both MBC sensitive and MBC resistant populations (Fitt, 1988). Resistant strains to prochloraz have been isolated on winter wheat in France (Poupard and Cavelier, 1992; Leroux and Gredt, 1997) but resistance to it has not developed in UK populations despite its extensive use (Fitt *et al.*, 1990). Analysis of *P. herpotrichoides* indicates that sensitivity to prochloraz may be under multigenic control (Dyer and Lucas, 1995; Dyer, Lucas and Peberdy, 1998). To obtain effective control, sprays need to be applied before lesions become severe and its was necessary to use MBC fungicides at growth stage 30/31. Prochloraz can give control when applied at growth stages 30 and 37 which gives growers the opportunity to delay treatment until the weather is optimal for spraying, or until the risk of a severe eyespot epidemic is more certain (Fitt *et al.,* 1990).

Cultural practices have been given added impetus since the occurrence of resistance to MBC fungicides. Late-sown crops show a reduced risk of severe eyespot epidemics (Yarham, 1986) because the R-type isolate develops more slowly in winter wheat and barley crops (Goulds and Fitt, 1988, 1990). Excessive use of nitrogen fertilisers and very high seed rates are both likely to produce lush crops and favour eyespot (Fitt, 1988). Intercropping may result in a secondary source for re-distribution of previously dispersed droplets and spores (Soleimani, Deadman and

McCartney, 1996). Straw incorporation has been found to reduce eyespot severity, perhaps due to competition from other faster growing fungi (Fitt, 1988). Stubble left to over-winter because of the ban on straw burning in England, the current policies on "set-aside" and rotations with spring sown crops may increase the incidence of the teleomorph of which the potential significance is still unclear.

2.1.10 Forecasting and detection

Visual diagnosis of eyespot is difficult especially when other diseases of the stem base such as *Fusarium* spp. are present. Yield losses are associated with severe lesions during grain filling and fungicides need to be applied early if they are to give effective control. Meteorological data has been use in forecasting conditions favourable for sporulation, dispersal and infection of eyespot on wheat in Germany (Rapidly *et al.,* 1979). When all the infection criteria such as temperature and humidity are fulfilled, lesion development occurs and a fungicide spray is recommended when it is predicted that the 4^{th} leaf sheath has been penetrated. The scheme does not allow for loss of lesions or temperatures below 0°C and the scheme has been found to be inappropriate in the UK (Fitt *et al.,* 1988). In 1986 (Anon) ADAS recommended that if at growth stage 30 more than 20% of leaf sheaths are infected at least to the second leaf sheath then a fungicidal spray should be used. This method assumes that all lesions will become damaging. However the relationship between symptoms at growth stage 30-31 is poorly related to the incidence at grain filling (Fitt, 1988). The development of diagnostic methods to identify *P. herpotrichoides* pre-symptomatically in the field using monoclonal and polyclonal antibodies in enzyme linked immunosorbent assays (ELISA) (Lind, 1990, 1992, Unger and Wolf, 1988; Priestly and Dewey, 1993) and to differentiate between W and R-types using PCR assays (Poupard *et al.,* 1993; Nicholson *et al.,* 1997) is now important for decision making in eyespot control. The use of ELISA assays (Lind, 1992) and recent development of competitive PCR assays (Nicholson *et al.,* 1997) now permits quantification of infection in cereals. These are also useful research tools as well as having practical application. Using this pathotype diagnostic

approach, colonisation of the hosts plants by the W and R-types can be investigated and factors such as population changes, host resistance and fungicide efficacy can be further explored experimentally.

2.2 Fungal pathogenicity factors

Fungal pathogenicity determinants are generally regarded as being necessary for disease development but not for normal growth on artificial media, although this distinction is arbitrary and not readily applicable to obligate pathogens. Knowledge of such determinants is valuable in developing understanding of the way fungal pathogens interact with their plant host and environment. Identification of key processes that may be targeted may give rise to new strategies for disease management. Three classes of pathogenicity factors will be considered in the present review: melanins, toxins and cell-wall degrading enzymes.

2.2.1 Melanin pigments

The dark brown to black pigments generically known as melanins are ubiquitous in nature and are produced by a variety of organisms including bacteria, fungi, plants and animals. These pigments enhance survival and competitive abilities of species in certain environments. In fungi, genera such as *Alternaria* Nees have pigmented conidia and hyphae (Bell and Wheeler, 1986). Other genera such as *Verticillium* Nees produce hyaline conidia and hyphae but have chlamydospores and microsclerotia that are heavily pigmented (Bell *et al.,* 1976b). Most genera have dark pigments in the sexual spores or their enclosing structures (Bell and Wheeler, 1986).

W and R-types of *P. herpotrichoides* are seen to grow *in vitro* with darkly pigmented hyphae. Studies on the infection plaques produced by W-types *in vitro* in agar culture and W and R-types on wheat seedlings found that the infection plaques consist of cells with thickened pigmented walls (Deacon, 1973; Daniels *et al.,* 1991, 1995). It has been observed that auxotrophic colour mutants appeared to be less pathogenic than wild-type strains (Hocart pers. com, 1987). However the characterisation and

role of melanin in *P. herpotrichoides* is currently undefined.

2.2.1.1 The role of melanin in fungi

Melanin pigments have been associated with fungal survival by allowing structures such as sclerotia, cleistothecia, chlamydospores and hyphae to be resistant to extreme environmental conditions including UV light, desiccation and temperature extremes (Durrell, 1964; Geis and Szaniszio, 1984; Linderman and Toussoun, 1966; Huang and Kokko, 1989; Jackson and Gay, 1976). Melanin also appears to be important in resistance to microbial lysis, in particular showing resistance to the hydrolytic enzymes chitinase, β-1,3- glucanase and β-1,6-glucanase produced by antagonistic fungi and bacteria found in the soil (Bloomfield and Alexander, 1967; Kuo and Alexander, 1967; Lockwood, 1960; Potgieter and Alexander, 1966).

Melanin has also been shown to be involved in pathogenicity. By using genetically defined melanin-deficient mutants melanin has been shown to be required for penetration of plant epidermis and of nitrocellulose membranes by the rice pathogen *Magnaporthe grisea* (Herbert) Barr. (anamorph *Pyricularia oryzae* Briosi & Cavara) (Chumley and Valent, 1990). The cucumber pathogen *Colletotrichum lagenarium* (Pass) Ell & Halsted (Kubo, Furusawa and Shishyama, 1987, Kubo *et al.*, 1982a, 1982b) and the bean pathogen *C. lindemuthianum* (Sacc. & Magn.) Scibner (Wolkow, Sisler and Vigil, 1983), form well developed melanised appressoria, but melanin is absent in the region of the appressorium pore from which the penetration peg emerges. Albino mutants form colourless appressoria and are non-pathogenic as they only germinate laterally to form secondary appressoria (Kubo *et al.*, 1982a; Chumley and Valent, 1990; Howard and Valent, 1996).

2.2.1.2 The location of melanins in fungi

Ultrastructure studies using wild-types and melanin deficient mutants of *C. lagenarium*, *M. grisea* and *Verticillium dahliae* Kleb. have determined the location of the electron dense melanin granules. Melanin granules which are involved directly in

pathogenicity in appressorial formation are found as an electron dense layer about 100nm thick positioned outside the plasmalemma (Fig 2.3) (Howard and Ferraria, 1989; Bourett and Howard, 1990; Kubo *et al.,* 1985; Kubo and Furusawa, 1986). In contrast melanin granules that are not involved in pathogenicity but in survival are associated with the cell wall and fibrillar network surrounding the wall (Fig 2.4) (Wheeler *et al.,*, 1976; Wheeler *et al.,* 1978). In albino and buff mutants or wild-types treated with the melanin-inhibiting compound tricyclazole, the fungal walls are electron translucent but normal melanin granules appear when the melanin pre-cursors scytalone and 1,8-dihydroxynaphthalene (DHN) respectively are added to the medium (Wheeler, Tolmsoff and Meola, 1976; Wheeler *et al.,* 1978; Bell and Wheeler, 1986).

A

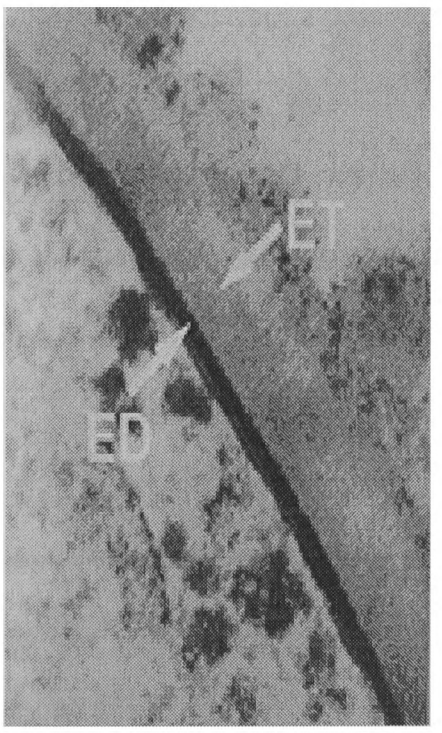


Fig 2.3: Appressorial cell wall of *C. lagenarium.* ED-electron dense layer (melanin granules), ET-electron transparent layer, both outside the plasmalemma magnification x 72 000. (Kubo and Furusawa, 1986)

B

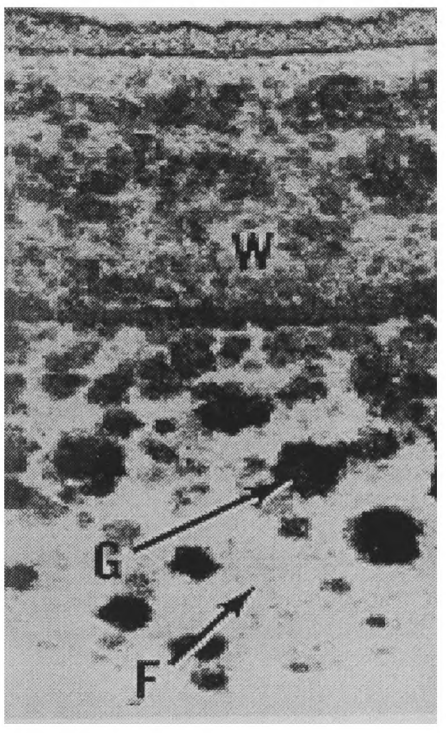


Fig 2.4: Microsclerotial cell wall of *V. dahliae*. G-melanin granule, F-fibrillar network external to the cell wall, W-cell wall magnification x 72 000. (Wheeler *et al.*, 1976)

2.2.1.3 Biosynthesis of fungal melanins

Melanins are of high-molecular weight and their black colour is due to their absorption of all visible wavelengths of light (Bell and Wheeler, 1986). Several different pathways have been identified for the biosynthesis of melanins in fungi. DOPA melanin is synthesised by the oxidation of tyrosine by the copper-containing enzyme, tyrosinase via 3,4-dihydroxyphenylalanine (DOPA). Tyrosinases from *A. nidulans*, *Neurospora crassa* Shear & Dodge and other fungi have been characterised (Bell and Wheeler, 1986). Melanins in cell walls of basidiomycetes are derived from γ-glutaminyl-3,4-dihydroxybenzene (GDHB) or catechol. GDHB is the precursor of spore wall melanin in the common mushroom *Agaricus bisporus* Lange (Stussi and Rast, 1981) and catechol is the precursor of melanin in teliospores of *Ustilago maydis* (DC.) Corda (Bell and Wheeler, 1986). Many Ascomycotina and related Deuteromycotina make melanin via the pentaketide pathway in which DHN is the intermediate precursor of the polymer (reviewed by Bell and Wheeler, 1986). The pentaketide pathway was first determined in *V. dahliae* using genetic mutants (Bell, Stipanovic and Puhalla, 1976a, Bell *et al.,* 1976b; Stipanovic and Bell, 1976, 1977) and the fungicide tricyclazole which inhibits the reductase enzymes in DHN melanin biosynthesis and is currently used to control rice blast disease (Tokousbalides and Sisler, 1979). Similar techniques have also been used to demonstrate this pathway in *C. lagenarium* (Kubo *et al.,* 1983), *M. grisea* (Woloshuk *et al.,*1980, 1982) and *Thielaviopsis basicola* Berk. & Broome (Stipanovic and Wheeler, 1980, Wheeler and Stipanovic, 1979). Figure 2.5 shows the DHN biosynthetic pathway. It starts from pentaketide synthesis and proceeds to form 1,3,6,8-tetrahydroxynaphthalene (1,3,6,8-THN). The next four steps alternate between reduction and dehydration i.e. the reduction of 1,3,6,8-THN to scytalone, dehydration of scytalone to 1,3,8-trihydroxynaphthalene (1,3,8-THN), reduction of 1,3,8-THN to vermelone and dehydration of vermelone to DHN. The enzymatic reductions require reduced nicotinamide adenine dinucleotide phosphate (NADPH) as a co-factor (Wheeler, 1982). DHN is then polymerised and oxidised to yield melanin.

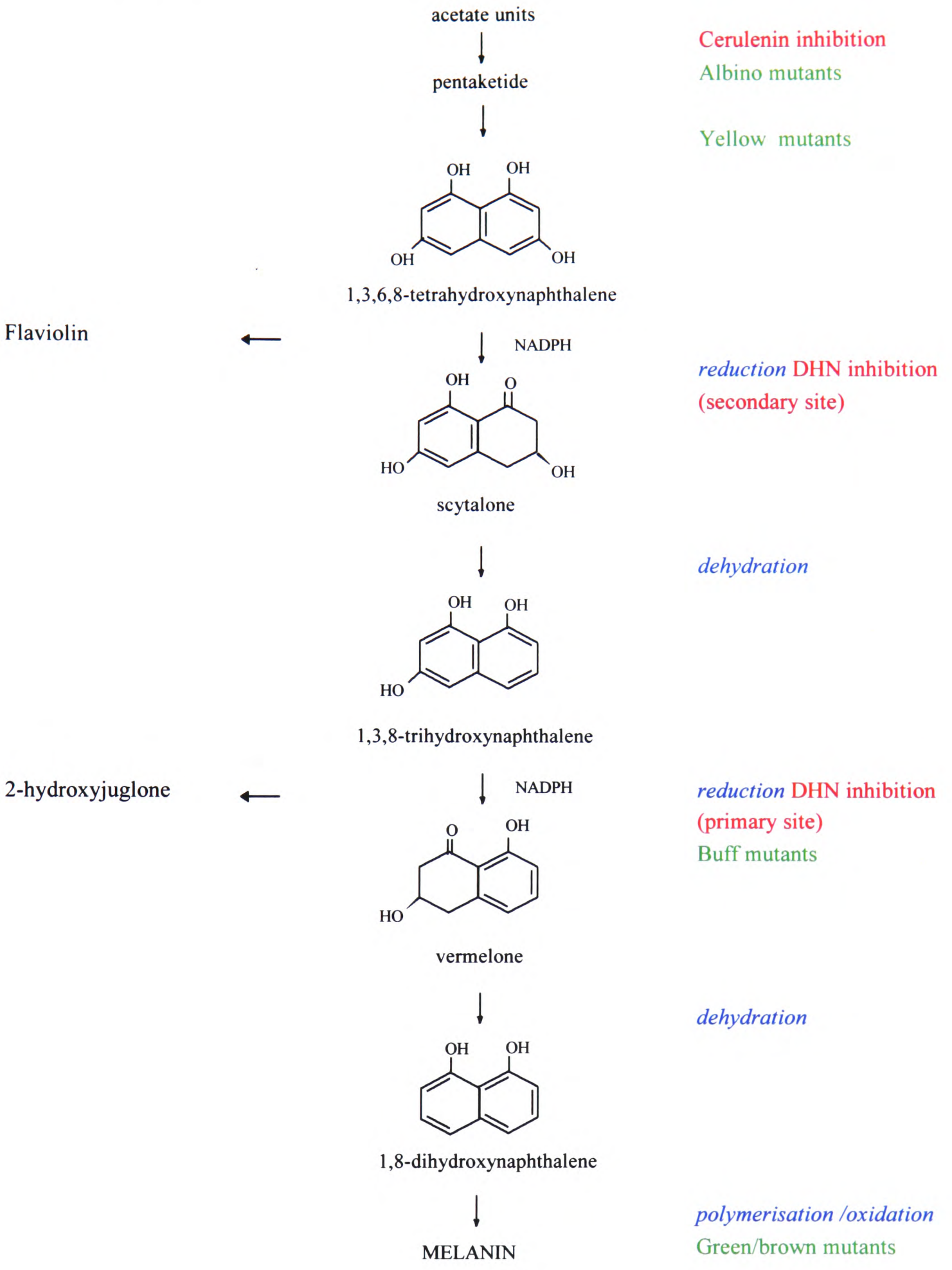


Figure 2.5: The DHN melanin biosynthetic pathway.

Yamamoto and Tsuru, 1985) and the antibiotic cerulenin inhibits the condensation reactions in the polymerisation of acetate and malonate in polyketide synthesis (Kubo *et al.*, 1986). These inhibitory compounds are used to verify the melanin biosynthetic pathway present.

The melanin biosynthetic pathway in *P. herpotrichoides* has not been established. However the colour mutants have been found to show reduced levels of pathogenicity (Hocart pers. comm, 1987). Understanding the role of melanin in the pathogenicity of *P. herpotrichoides* and determining its biosynthetic pathway may lead to possible target sites for controlling this disease.

2.2.2 Chemical mechanisms of pathogenicity

When in close proximity to the host cell wall many pathogenic fungi launch biochemical attacks. Many complex interactions occur resulting in cellular damage and the development of disease symptoms. Two of the classes of chemical weapons include high molecular weight cell wall degrading enzymes which soften and rupture cells on contact, leading to tissue maceration, and low molecular weight toxins which kill cells in advance of invasion into host tissue.

2.2.2.1 Enzymatic digestion of the plant cell wall

The plant cell wall is a complex, three-dimensional barrier composed of integrated cross-linked polymers. It is growing and constantly changing structure for which the constituents and their relative proportions may vary during growth. Cell wall degrading enzymes are of high molecular weight and are important in the penetration and spread of the fungus within host tissues. Most but not all pathogens can produce extracellular cell wall degrading enzymes capable of degrading the major glycosidic linkages in plant cell walls. Penetration into plant tissues is brought about by the penetration of the cuticle which consists primarily of cutin and may be impregnated with wax and often covered by a wax layer. The primary cell wall and in certain specialised cells a secondary wall consist of a microfibrillar phase and a matrix

phase. The microfibrillar phase is composed of microfibrils made up of cellulose, an unbranched β1,4-glucan and often mannose and xylose are present. The matrix phase consists of a variety of polysaccharides, proteins and phenolics. Extraction of the polysaccharides yields a fraction known as pectin and a fraction known as hemicellulose (reviewed by Brett and Waldron, 1990).

Pectic polysaccharides are present in the middle lamella and primary wall of monocotyledonous plants although to a lesser extent than is found in dicotyledonous ones. The key polymer is rhamnogalacturonan, which comprises chains of α-1,4-linked galacturonic residues interspersed with β-1,2-linked rhamnose. The carboxyl groups of the galacturonic acid may be methylated or cross linked with calcium. Arabinose and galactose side chains may be attached to the rhamnose. Hemicelluloses primarily consist of xylan which has a backbone of β1,4-linked xylose. In monocotyledon primary walls arabinose is a dominant side chain giving rise to arabinoxylan. Glucomannans, xyloglucan, mannans and galactomannans may also be present.

The cell-wall degrading enzymes consist of pectic enzymes, polygalacturonase (PG) and pectin lyases (PL) which can split the galacturonic chains forming unsaturated bonds and pectin methyl esterase (PME) which removes methyl groups. Both PG and PL can be subdivided into *endo* or *exo* forms which attack the chains randomly or terminally respectively (Cooper, 1983, 1984). The ability to produce pectic enzymes has been suggested to contribute to the virulence of isolates of *F. oxysporum* f.sp *elaeidis* Schl. (Osagie and Obuekwe, 1991) and *V. albo-atrum* Reinke & Berthold (Durrands and Cooper, 1988). The other polymer degrading enzymes include cutinase, xylanase, glucanase, galactanase, arabinase, mannase and cellulases (c1, c2, Cx, β-glucosidase) (Cooper, 1984; Oliver and Osbourn, 1995). The production of these enzymes can lead to tissue maceration, leakage of ions due to degradation of the cell walls which support the limiting plasma membrane and thus aid nutrition and colonisation of the host by the pathogen. Pectolytic enzymes, cellulases, xylanase

and arabinase are known to be secreted by *P. herpotrichoides in vitro* in liquid culture and *in vivo* when infecting wheat (Mbwaga, Menke and Grossmann, 1997; Cooper *et al.*, 1988; Hanssler, Menke and Grossmann, 1971). The correlation between the production of these enzymes and their role in pathogenicity has not yet been demonstrated, but the W x R recombinant hybrids which vary in their pathogenicity may be useful tools to do this with.

2.2.2.2 Toxins in pathogenicity

In certain plant diseases a proportion of the syndrome may be caused by toxic substances produced by the host pathogen. A toxin can be defined as a compound probably of low molecular weight, produced by a microbial pathogen which causes damage to the host plant and which is known to be involved in the development of plant disease (Scheffer, 1983). Killing cells in advance of invasion, and leakage of electrolytes and nutrient materials from the host plant cells, may be an advantage to the pathogen. Alternatively toxins may upset the normal metabolism of the host plant and suppress resistant mechanisms which would otherwise be induced, thus enabling colonisation to occur with little resistance.

Toxins are usually classified as host selective or non-selective (Scheffer, 1983). Host selective toxins are required for pathogenicity and determine the host range of the producing fungi. *A. kikuchiana* Tanaka, the agent of the black spot disease of Japanese pear, was first shown to produce culture filtrates that had host specific toxicity (Pringle and Scheffer, 1964). Other host specific toxins have since been identified, examples of which are produced from *Cochliobolus (Helminthosporium) victoriae* Nelson (Meeham & Murry), victorin (HV) toxin affecting oats (Scheffer and Ullstrup, 1965); *Bipolaris (Helminthosporium) sacchari* (B. de Han) Butl., helminthosporoside (HS) toxin affecting sugar cane (Steiner and Byther, 1971); and *Periconia circinata* (Mangin) Sacc., PC toxin affecting sorghum (Scheffer and Pringle, 1961). The known host specific toxins produce all the symptoms of the disease caused by the respective pathogen. Symptoms associated with various toxin

action include loss of electrolytes (Damann, Gardner and Scheffer, 1974), changes in membrane potential and respiration (Scheffer and Pringle, 1963) and induction of ultrastructural changes in mitochondria (Gilchrist, 1983). Correlations have been shown to exist between toxin producing ability and pathogenicity in many host specific toxin-producing fungi (Luke and Wheeler, 1955; Scheffer and Pringle, 1961; Scheffer, Nelson and Pringle, 1964).

Host non-specific toxins have been shown to produce all or part of the disease syndrome, not only on the host plant but also on other species of plant that are not normally attacked by the pathogen in nature. In contrast to host specific toxins, host non-specific toxins are not required for pathogenicity but contribute to virulence and are responsible for certain symptoms.

Examples of non-specific toxins include tabtoxin, produced by *Pseudomonas syringae* pv. *tabaci* (Wolf & Foster) Young *et al* causing wildfire disease of tobacco and fusicoccin, produced by *F. amygdali* causing twig blight on almond and peach trees (Scheffer, 1983). A correlation between the ability to produce the toxin phaseolotoxin and cause chlorosis in beans has been found with *P. syringae* pv. *phaseolicola* (Burkholder) Young *et al* the causative agent of bean blight (Scheffer, 1983). Tab toxin and phaseolotoxin have been shown to affect the cellular transport system H^+/K^+ pumps in cells and enzyme activity (Gilchrist, 1983).

Girko *et al,* (1993) suggested that *P. herpotrichoides* produces toxic substances when grown in liquid culture. In addition to this observation, an examination of the lesions on the host stem bases which are a symptom of the disease found that host cell death occurred several cells away from the fungus indicating that perhaps a small molecule may be inducing cell death in advance of hyphal infection (Hocart pers. comm, 1986).

Detecting and determining the role of possible toxins involved in pathogenicity of

P. herpotrichoides using the W x R hybrids which vary in their pathogenic ability and thus ability to cause disease symptoms combined with the role of cell-wall degrading enzymes and melanin, will help identify mechanisms involved in the pathogenicity of this fungus.

Plate 2.1

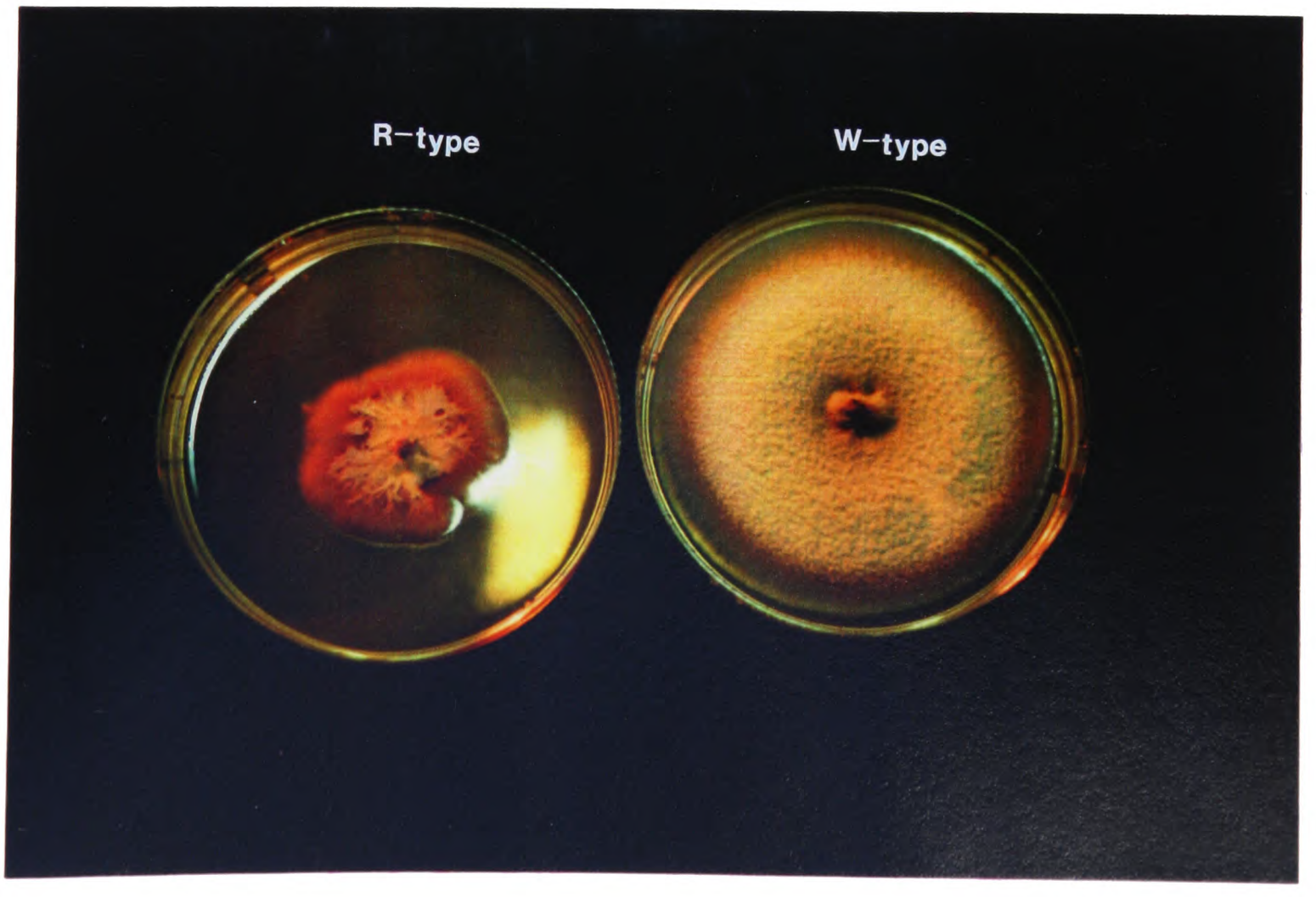
Disease symptoms caused by *P. herpotrichoides* and W and R-type colony morphology on MYG agar.

A) Disease symptoms caused by *P. herpotrichoides* on wheat seedlings.

B) Colony morphology of W and R-types of *P. herpotrichoides* on MYG agar.

A

B



CHAPTER 3

GENERAL MATERIALS AND METHODS

3.1 Strains and hybrids

The strains and hybrids of *P. herpotrichoides* used in the experimental work are summarised in Tables 3.1 and 3.2.

Table 3.1: Field strains and the source from which they were obtained for experimental work.

***P. herpotrichoides* Strain code**	**Description**	**Source**
22-8	R-type	M. Hocart, SAC, Edinburgh
22-119	R-type	M. Hocart, SAC, Edinburgh
23-2	R-type	M. Hocart, SAC, Edinburgh
22-12	R-type	M. Hocart, SAC, Edinburgh
22-1	W-type	M. Hocart, SAC, Edinburgh
22-2	W-type	M. Hocart, SAC, Edinburgh
22-20	W-type	M. Hocart, SAC, Edinburgh
C87/631/1	W-type	P. Nicholson, John Innes centre, Norwich
C78/501	W-type	P. Nicholson, John Innes centre, Norwich
C91/761	W-type	P. Nicholson, John Innes centre, Norwich
22-433	W-type	P. Dyer, University of Nottingham, Nottingham
24-1	*P. anguioides*	M. Hocart, SAC, Edinburgh

Table 3.2: W x R hybrids, inter-specific hybrids and their parental strains.

Parental strains	**Hybrid progeny**
R-type 23-2 and W-type 22-20	D2/35, D2/36, D3/41, D3/48, D5/82, D5/83b, D5/100, H3/1 FPA HLM, H1/1 FPA HLM
R-type 22-8 and W-type 22-20	C41, P262, O251, AE586, AE588, AE591, A12, B27, D62, E75, F90, G109, 37I, 128I, I131, 138I, J160, J166, M218, X397, Z449a, Z449o, AI382, AI386, AI387
R-type 22-12 and W-type 22-20	G12/4 CH HLC, G12/4 CH MM
R-type 22-12 and *P. anguioides* 24-1	AJ, DQ, DK, DM, EP, FG

3.2 Maintenance of cultures

All cultures were maintained on malt, yeast, glucose agar (MYG) (g l^{-1}, malt extract, 5; yeast extract, 2.5; glucose, 10; agar 16;) in 9cm diameter plastic Petri-dishes. Cultures were point inoculated from 14 day old plates, sealed with parafilm and stored upside down to allow condensation to collect in the Petri-dish lid. Growth in liquid culture consisted of 40ml MYG, omitting the agar, in 250ml conical flasks. Flasks were inoculated by scraping 14 day old MYG plates with a round blade scalpel in 2ml sterile distilled water (SDW). Mycelial fragments were then added to the flasks using a sterile Pasteur pipette, the flasks were sealed with a foam bung and placed on at rotary shaker at 100rpm. Unless stated, cultures were always incubated in the dark at 19°C.

All media was sterilised in an autoclave at 121°C, 2.2 bar for 20 minutes and allowed to cool before pouring or being inoculated.

3.3 Stock cultures

Stock cultures were prepared by point inoculation from 14 day old MYG plates on to 1ml MYG agar slants in 2ml cryo-vials. The cultures incubated at 19°C in the dark for 3 weeks and were frozen to -80°C before being stored in liquid nitrogen.

Stock cultures were also prepared by point inoculation from 14 day old MYG plates on to 5ml MYG agar slants in 10ml plastic universal bottles. The cultures were left to grow at 19°C in the dark for 3 weeks before being sealed with Parafilm and stored at 4°C.

3.4 Production of conidia

Conidia were produced on tap water agar (TWA) (g l^{-1}, agar 16). Mycelial plugs were placed on the agar and the plates were incubated at 16°C for 10 days under near UV light (wavelength 360nm). When large numbers of conidia were required they were produced by the method of Reinecke and Fokkema (1979). Fourteen day old TWA

plates were scraped in 2ml SDW. Approximately 1ml was removed containing 1 x 10^6 spores and spread across a 9cm MYG plate. The MYG plates were incubated at 19°C in the dark for 2-3 days. Microcyclic conidia resulted in a cream coloured slurry, of conidia on the surface of the agar. All conidia were collected by suspension in SDW and filtered through 2 layers of muslin to remove mycelial fragments. Conidia were washed twice in SDW by centrifugation (3000*g* for 10 min) before use.

CHAPTER 4

CHARACTERISATION OF FIELD STRAINS AND PARASEXUAL RECOMBINANT HYBRIDS AND THE DETECTION OF SECONDARY METABOLITES

4.1 Introduction

Traditional detection and identification of strains of *P. herpotrichoides* have used systems based on colony morphology, pigmentation on maize meal agar, pathogenicity to wheat and rye, and conidial morphology (King and Griffin, 1985; Sanders *et al.,* 1986; Creighton, 1989; Lange de la Camp, 1966; Scott *et al.,* 1975; Nirenberg, 1981). Correlation between these systems has been found however to be incomplete (Hollins *et al.,* 1985; Fitt *et al.,* 1987; Creighton, 1989). Molecular techniques using RAPDs, RFLPs and specific primers (which have been based on previously characterised W and R-types by traditional systems) now give more rapid and reliable identification systems for W and R-types (Nicholson *et al.,* 1991b, 1997; Poupard *et al.,* 1993; Takeuchi and Kuninaga, 1996; Nicholson and Rezanoor, 1994). The use of the parasexual cycle has produced W x R and inter-specific recombinant hybrids. The pathogenicity of these hybrids to wheat, barley and rye has lead to the suggestion that control of pathogenicity is separate for these hosts. Some hybrids had also being given the classification of being asymptomatic, thus able to infect host stem bases without causing disease symptoms (Hocart and McNaughton, 1994; McNaughton, 1996). The aim of this work was to use cultural, pathogenicity and molecular techniques to characterise strains and parasexual hybrids. The ability of these strains and hybrids to produce infection plaques *in vitro* was then examined and related to their pathogenicity on wheat, barley and rye. Finally, an investigation was made using selected strains and hybrids which were classed as pathogenic, asymptomatic or non-pathogenic on wheat, barley and rye to detect for the presence of any secondary metabolites (toxins, enzymes or elicitors) which may have been involved in pathogenicity and disease symptom induction.

4.2 Materials and Methods

4.2.1 Strains and hybrids

The field strains, W x R hybrids and inter-specific hybrids that were used in the work reported in this chapter are described in Tables 3.1 and 3.2 and summarised in Table 4.1.

Table 4.1: Strains and hybrids used for characterisation.

Parental/field strains	Number of hybrid progeny
W-types 22-1 and 22-2	
R-type 22-119	
R-type 23-2 and W-type 22-20	9
R-type 22-8 and W-type 22-20	25
R-type 22-12 and W-type 22-20	2
R-type 22-12 and *P. anguioides* 24-1	6

4.2.2 Growth rate and colony morphology on MYG agar

Strains and hybrids were point inoculated on to 9cm diameter Petri-dishes containing MYG agar and incubated for 14 days before an assessment was made of the growth rate (mean of 2 perpendicular measurements of colony diameter/days growth) and colony morphology. Three replicate plates were used per strain/hybrid and the growth rate data was analysed using a single factor analysis of variance (ANOVA).

4.2.3 Colour pigmentation on maize meal agar

Strains and hybrids were point inoculated on to 9cm diameter Petri-dishes containing maize meal agar (g l^{-1}, maize meal, 40; agar, 15) and incubated under near UV light (wavelength 360nm) for 14 days before an assessment was made on colour pigmentation using a colour identification chart of Flora of British Fungi, Royal Botanic Garden Edinburgh. The assessment was made using the underside of each Petri-dish. Each strain/hybrid was replicated 3 times.

4.2.4 Production of conidia

Conidia were produced on TWA as described in Section 3.4. After 14 days incubation, the ability to produce conidia and conidial morphology (shape and number of cross walls) was assessed. Four agar plates inoculated with hyphal plugs were examined for each strain/hybrid.

4.2.5 Growth of cultures for molecular characterisation

Selected W and R strains and hybrids listed in Table 4.4 were used for DNA characterisation due a limitation on resources. Conical flasks (250ml) containing 40ml MYG (omitting the agar) were inoculated with aerial mycelium scraped using a sterile round blade scalpel and 1-2ml SDW from 14 day old colonies grown on MYG agar. The flasks were sealed with a foam bung and incubated on an orbital shaker at 100 revolutions per minute (rpm) at 19°C for 14 days in the dark. Mycelium was removed from the flasks by vacuum filtration using Whatman filter paper (grade 181), and collected in plastic Petri-dishes. The mycelium was frozen at -70°C for 1 hour before being freeze dried (-55°C, 10^{-1} Torr) for 24 hours. Freeze drying prevented degradation of the samples.

4.2.6 DNA Extraction

The freeze dried hyphae were placed into pre-weighed universal bottles and milled using 2 steel ball bearings (diameter 6mm) on a vortex mixer until a fine powder was obtained. The ball bearings were removed and the milled material was transferred to 15ml disposable centrifuge tubes.

Extraction of the DNA was achieved by using hexadecyltrimethylammonium bromide (CTAB) buffer (6ml added to the milled material in 15ml centrifuge tubes). The tubes were incubated at 65°C for 90 minutes, being shaken at intervals. Following incubation 2ml of 5M potassium acetate and 1.5ml $CHCl_3$ were added ($CHCl_3$ helps precipitate the lipid layer leading to cleaner samples) and mixed by inversion before being placed at -20°C for 30 minutes. The tubes were then

centrifuged at 3000rpm for 15 minutes. After centrifuging, 5ml of supernatant were removed from each and added to further 15ml tubes containing 10ml of ice-cold ethanol to precipitate the DNA. These tubes were left to stand for 15 minutes at room temperature before centrifugation at 659 x g for 10 minutes to precipitate the DNA pellet. Each pellet was washed in 1ml of ice-cold 70% ethanol and centrifuged at 659 x g for 10 minutes. The washing process was repeated to ensure the removal of salts and the pellets then left to air dry. Once dry, the pellets were re-dissolved in 500 µl tris EDTA (TE) buffer pH8. All DNA extractions were stored at 4°C until required for use.

4.2.7 PCR amplification

Amplification reactions were carried by the method described by Nicholson *et al.* (1996) in volumes of 50µl containing 2µl of a 10 fold stock dilution of DNA. The reaction buffer consisted of 100µM each dATP, dCTP, dGTP, dTTP, 100nM each forward and reverse primer and 2 units Taq polymerase (Dynazyme, Flowgen) in 10mMTris HCl (pH8.3), 1.5mM $MgCl_2$, 50mM KCl, 100µg ml^{-1} gelatine, 0.05% Tween 20 and 0.05% Nonidet P-40. Reaction mixtures were overlaid with mineral oil prior to PCR. Specific primers Ty16F/R and TaO5F/R were used to identify W and R-types respectively (Nicholson *et al.,* 1997). Non specific primers ITS4 and ITS5 were used identify the internal transcribed spacer (ITS) region of the nuclear ribosomal (r)DNA (White *et al.,* 1990) (supplied by PE Applied Biosystems).

Amplification was carried out in a Perkin-Elmer Cetus 480 Thermal Cycler. Touchdown PCR was used to ensure specificity of the reaction (Don *et al.,* 1991). The first segment was at 95°C for 1 minute to achieve denaturation of the DNA. An annealing step at 66°C for 20 seconds was used for the first 5 cycles of PCR. This was followed by 5 cycles at 64°C annealing then 30 cycles at 62°C annealing. A denaturation step at 95°C for 30 seconds was used throughout, along with an extension step at 72°C for 45 seconds. The maximum ramping rates of the PCR machine were used with the default setting being at 1°C S^{-1}. A final extension step of

5 minutes at 70°C followed and the reactions were held at 10°C until removal from the machine.

Following amplification, the PCR products of each reaction were separated by electrophoresis through 1.5% agarose gel (10 volts cm^{-1} gel length for 90 minutes) using tris-acetate EDTA (TAE) buffer with a DNA 1.22kb ladder molecular weight marker. Gels were stained with ethidium bromide (1nM ml^{-1}) and photographed under UV illumination on a 'Gel Doc 1000' system (Bio-Rad).

4.2.8 Restriction digest

Aliquots of 20µl of ITS product were incubated with 16µl SDW, 4µl reaction buffer (supplied with enzyme) and 0.2µl *Hae III* enzyme (Boehringer Mannheim) at 37°C overnight. The restriction fragments (19µl digest + 1µl bromophenol blue loading dye) were separated by electrophoresis through a 2% agarose gel using a TAE buffer with a DNA 1.22kb ladder molecular weight (running conditions as above). Gels were visualised and stained as described in Section 4.2.7.

4.2.9 Growth of test plants

Ten seeds of wheat cv. Beaver, barley cv. Halcyon and rye cv. Halo, were sown in 12.5 cm pots filled to within 2 cm of the rim with peat based compost. These were placed in a glasshouse for approximately 3-4 weeks until they had reach growth stage 11-12 (1 ½ leaves).

4.2.10 Preparation of fungal inoculum

Fungal inoculum was produced by growth of the strains and hybrids on MYG agar for 2 weeks in 9 cm diameter plastic Petri-dishes. Conical flasks (250ml) containing 100ml malt extract medium, 25g l^{-1} were inoculated with aerial mycelium scraped using a round bladed scalpel and 1-2ml SDW from 14 day old colonies grown on MYG agar. The flasks were sealed with a foam bung and incubated in the dark at 19°C being shaken daily to aerate and distribute the hyphal fragments.

Mushroom spawning bags (Van Leer Ltd) were cut to 28 x 20 cm in size, containing a perforation area of 26 x 16cm, to which 200g of untreated oat grain were added. Tap water (100ml) was added and the bags left for 24 hours to allow the grain to absorb the water enabling better penetration of the heat into the grain when autoclaved and for the germination of any fungal spores present. The grain was autoclaved twice on consecutive days at 121°C for 30 minutes. The bags were inoculated by pouring inside the 10 day old fungal cultures which had been grown in malt extract medium. They were then sealed with tape and incubated lying flat in the dark at 19°C for 8 weeks (Plate 4.1). After 4 weeks additional SDW (100ml) was added to those bags that had dried out.

4.2.11 Inoculation of test plants

The pathogenicity of all strains and hybrids (described in Section 4.2.1) were tested. Inoculation of the plants was done by placing a small amount of inoculum around the stem base of each plant, making sure that the grain always touched the stems to ensure that infection would occur. The pots were filled with vermiculite to maintain a high humidity around the stem base needed for infection (Plate 4.1). The plants were then grown for 8 weeks in an unheated glasshouse and watered from below using capillary matting to avoid cross contamination between pots. Three sets of oat grain inoculated with 100ml malt extract (25g l^{-1}) were used as controls. Each pot contained 8 plants and three pots (24 seedlings) were used for each strain and hybrid on each cereal species. The pots were assigned randomly in a 3 block design.

The experiment was conducted in the winter months at the Bush Estate, Roslin near Edinburgh. The minimum and maximum air temperature was measured with a thermohydrograph placed next to the plants. The mean day temperature (highest recorded) was 17.1 $\pm$ 0.6°C and the mean night temperature (lowest recorded) was 2.7 $\pm$ 2.6°C.

4.2.12 Pathogenicity assessment

Assessment of pathogenicity was made visually using the infection scoring system described by Scott (1971). Uninfected plants scored 0; coleoptile infected, 1; coleoptile penetrated, 2; first leaf sheath infected, 3; first leaf sheath penetrated, 4; second leaf sheath infected, 5; second leaf sheath penetrated; and so on.

4.2.13 Microscopic examination

An assessment was made for the presence of infection plaques by removing leaf sheaths from 2 stems from each pot, and staining all surface structures with a fluorochrome. The first, second and third leaf sheaths were placed on glass microscope slides and a few drops of freshly prepared 70% ethanol added to fix them. The tissue was then mounted in a few drops of a 0.01% solution of 8-anilino-sulphonic acid (ANS) made up in 0.05M phosphate buffer pH5 (0.93g citric acid and 2.12g potassium hydrogen phosphate in 100ml adjusted to pH 5 with 4M sulphuric acid). After 5 minutes the slides were examined for infection plaques under UV microscopy using a Leitz orthoplan II microscope fitted with a 100W mercury vapour lamp, a dichromic mirror and Biener filters. The abundance of infection plaques was scored separately for the three leaf sheaths examined on a scale of 0-5 as described in Table 4.2.

Table 4.2: Scoring scale for infection plaque production from each sample assessed

Abundance score	Number of infection plaques seen
0	0
1	1-25
2	26-50
3	51-75
4	76-100
5	>100

4.2.14 Data analysis

To test for differences in pathogenicity between strains the infection score data was analysed using the Kruskal-Wallis analysis of variance by ranks (Zar, 1974).

This test assumes that the data is non-parametric i.e. assumptions have not been made about the distribution of the sample population, such as it being normal or having equal variances. It is appropriate when the data is based on an ordinal scale derived from the infection scoring system used here. The 24 score values for each strain (3 replicates x 8 plants per pot) were placed in ascending order before assigning each data point a rank value.

To test for significant differences in pathogenicity between isolates the Kruskal-Wallis test statistic *H* was used

$$H = [12/N(N+1)]\Sigma R^{2}_{i}/n_i - 3(N+1)$$

where n_i is the number of observations in group *i*, N is the total number of observations in all groups, and R_i is the sum of the ranks of the n_i observations in group *i*.

To take into account tied ranks a correction factor was computed as

$$C = 1-\Sigma t/(N^{3} - N)$$

Where,

$$\Sigma t = m_i\Sigma(t^{3}_{i} - t_i)$$

t_i is the number of ties in the *i*th group of ties, and *m* is the number of groups of tied ranks.

The corrected value of *H* was then

$$H_c = H/C$$

The critical value for these tests was taken from the Table χ^2 with k - 1 degrees of freedom where k is the total number of strains included in each analysis.

The data was analysed separately for wheat, barley and rye. An initial analysis tested for any differences within the whole data, a further analysis tested for differences between strains (omitting un-inoculated controls) and finally an analysis was carried out to test for differences between pathogenic strains (a disease infection score greater than zero).

A blocking effect was tested for using a 2 factor ANOVA with replication of the data transformed with $\sqrt{(X+0.5)}$ to ensure homogeneity of the variances.

4.2.15 Infection plaque assay

The ability of the strains and hybrids to produce infection plaques *in vitro* was determined. A preliminary study tested various surfaces in combination with different media strengths as described in Table 4.3 using strains 23-2 and 22-119. The surface under test was placed on the MYG agar after it had cooled. The strains were point inoculated near or on to the surface of interest and incubated at 19°C. Assessments were made every 5 days for the production of infection plaques.

Table 4.3: Surfaces and media strength tested with strains 23-2 and 22-119 for the ability to induce infection plaques.

Surface	MYG agar Strength
MYG agar in Petri-dish	Full
Scratched MYG agar using a sterile cocktail stick	Full
Filter paper placed flat on MYG agar	Full
Cheese cloth placed flat on MYG agar	Full
A glass slide placed flat on MYG agar	Full and Half
A scratched glass slide using a sterile scalpel placed flat on MYG agar	Full and Half
Sloped MYG agar in a glass Petri-dish	Full and Half
Slope MYG agar in a plastic Petri-dish	Full and Half
Broken plastic placed flat on MYG agar	Full and Half
Cellophane placed flat on MYG agar	Full and Half
A plastic cover-slip placed flat on MYG agar	Full and Half

Following this preliminary study the ability of all the strains and hybrids (described in Section 4.2.1) to produce infection plaques was determined using glass Petri-dishes and full strength MYG agar. Sterile glass Petri-dishes (9cm diameter) were filled with 7ml MYG agar (plus streptomycin sulphate 100mg l^{-1} and chloramphenicol 50mg l^{-1}). The Petri-dishes were set at an angle of 45°C. A $1cm^2$ plug of inoculum from 14 day old cultures grown on MYG agar was placed 1cm from the lower edge of the sloped MYG agar. The dishes were then incubated at 19° C. Each strain /hybrid was replicated 3 times in separate plates. Every 7 days the cultures were examined for the presence of the hyphal front grown off the agar slope and on to the bottom of the glass Petri-dish where infection plaques should form.

Once adequate growth had occurred measurements were made for:

1) The number of infection plaques per 10 graticules. One graticule = 0.25 x 0.25mm (counted just after the edge of the slope).
2) The size of the infection plaques in relation to a single graticule.

Due to the difference in growth rates of the strains and hybrids, measurements took place from 6 to 15 weeks after inoculation.

4.2.16 Detection of secondary metabolites

A wheat cell suspension assay and a wheat root growth inhibition assay were developed to detect secondary metabolites (toxins, enzymes, elicitors) detrimental to wheat cells which may have been secreted into fungal culture filtrates.

4.2.16.1 Strains and hybrids

The R-type strain 22-8 and inter-specific hybrid DK were used for preliminary studies to produce culture filtrates when grown in MYG and in a minimal media containing wheat cell wall extracts. This strain and hybrid were chosen because their high pathogenicity produced strong disease symptoms on wheat (seen previously in

examination of their pathogenicity on wheat, barley and rye). Strain 22-20 and the W x R hybrids P262, O251 and C41 were used in further studies to produce culture filtrates when grown only in MYG, because of their lower pathogenicity, being asymptomatic or non-pathogenic on wheat (detected previously). No other strains or hybrids were tested as no conclusive results were obtained from the preliminary or further studies.

4.2.16.2 Production of fungal culture filtrates in MYG

MYG was chosen as the culture medium because of known ample hyphal growth in this medium (Plate 4.4). Culture filtrates were produced by inoculating conical flasks (100ml) containing 10ml MYG (omitting the agar) with 3 agar plugs taken from the growing edge of 14 day old cultures grown on MYG agar using a 4mm diameter cork borer. Flasks were sealed with 2 layers of aluminium foil, before being placed on a rotary shaker at 100 rpm at 19°C in the dark for 30 days. Controls flasks were un-inoculated.

Sampling of the flasks took place every 3 days from 2 replicate flasks each time up to 30 days post inoculation. In later studies with strain 22-20 and W x R hybrids P262, O251 and C41 sampling took place every 3 days starting from 18 days after inoculation.

4.2.16.3 Production of fungal culture filtrates in minimal media containing wheat cell walls

A minimal medium containing plant cell walls was used in comparison to the complete MYG medium because autocatalytic factors are often required to induce enzyme production. Only a minimal amount of nutrients was present because low nutrient levels may also serve to induce secondary metabolite production (Plate 4.4).

Plant cell wall extractions occurred by the modification of the method described by Karr and Albersheim (1970). Stem bases were taken from 14 day old wheat cv.

Beaver seedlings grown in a peat based compost in a glass house (8 hour day, approximately 25°C day temp, 8°C night temp). The wheat plants were placed in the dark for 48 hours to ensure that starch would not contaminate the cell wall prior to excising the stem bases. The excised stem bases were then frozen at -20°C before being ground into a fine powder in liquid nitrogen. The frozen powder was weighed and then placed in a blender containing 2.5 volumes (v/w) of cold 0.1M potassium phosphate buffer pH7. The insoluble material was collected on 2 layers of cheese cloth and the residue re-suspended in 1 volume (v/w) of the same cold buffer, the suspension was then left to stand for 5 minutes with occasional stirring before being filtered through 2 layers of cheese cloth. This washing process was repeated 4 times. Following the buffer washes the residue was washed once with SDW to remove salts. The residue was then suspended in 2.5 volumes of a cold mixture of chloroform and methanol (1:1 v/v) and ground for 5 minutes in the blender. The insoluble material was collected on 2 layers of cheese cloth by filtration and washed 3 times with 1 volume of the chloroform methanol mixture. The residue was then washed 3 times with 1 volume of acetone. The residue consisting of the cell walls was then air dried at room temperature, ground to a fine powder in a blender and stored at room temperature. All extractions up to the chloroform-methanol phase were carried out at 4°C. The latter extractions from and including the chloroform-methanol phase were carried out in a fume hood at room temperature.

The minimal medium to which the cell walls were added consisted of g l^{-1}, potassium hydrogen phosphate, 0.1; sodium nitrate, 0.2; magnesium sulphate ($7H_2O$), 0.5; potassium chloride, 0.5; sodium chloride, 0.5; calcium chloride, 0.5; thiamine-HCl, 1ml; trace elements, 1ml. The trace element solution consisted of mg l^{-1}, $Na_2B_4O.7H_2O$, 0.01; $CuSO_4.5H_2O$, 0.1; $FePO_4.2H_2O$, 0.2; $MnSO_4.5H_2O$, 0.2; $Na_2MoO_4.2H_2O$, 0.02; $ZnSO_4.7H_2O$, 2.0.

100ml flasks containing 10ml of minimal media with 0.06g cell wall extract were inoculated with strain 22-8 and the W x R hybrid DK. These flasks were inoculated,

incubated and sampled as for MYG fungal culture filtrates described above with the same number of replicates.

4.2.16.4 Purification of culture filtrates

The culture filtrates were filtered through 2 layers of cheese cloth and then centrifuged at 20000 x g for 10 minutes to remove hyphal fragments and wheat cell walls. The filtrates were then dialysed in 100 times the volume of de-ionised water for 24 hours at 4°C to remove sugars before being filter sterilised through sterile 0.2μm membrane filters and stored at 4°C in sterile universal tubes (10ml) until assayed.

4.2.16.5 Wheat cell suspension cultures

Wheat cell suspension cultures were obtained from Richard Brettle CSIRO Division of Plant Industry, Canberra. The L1 cell line used came from wheat cv. Vilmorin and contains a pair of chromosomes from *Thinopyrum intermedium* (Brettell *et al.,* 1988).

4.2.16.6 Maintenance of cell suspension cultures

Wheat cells were sub-cultured every 7 days by the removal of 3ml of the cell suspension into 6ml fresh medium in 100ml flasks. The cell culture medium comprised l^{-1}, 1 packet of M & S salts (basal) (Murashige and Skoog, 1962); sucrose, 20g; 0.5mg ml^{-1} 2-4D, 4ml; coconut water (Sigma Co. Ltd), 20ml; casein hydrolysate (Sigma Co. Ltd), 50mg; glucose, 10g; mannitol, 20g and kinetin, 0.2mg. This was adjusted to pH5.8 with KOH before autoclaving. The flasks were sealed with 2 layers of aluminium foil. Cell suspension cultures were incubated on a rotary shaker at 100rpm at 25°C with room light provided by 70 watt, cool white fluorescent tubes.

Preliminary studies monitored the daily increase in fresh and dry weight and change in pH over 10 days growth. These parameters were assessed by sub-culturing from a single flask, to maintain similar cell numbers and viability, into 3 replicate flasks per assessment. Fresh weight was measured using wetted pre-weighed cheese cloth. The

cell suspensions were then filtered through the cloth and the cloth re-weighed. Dry weight was measured using pre-dried (50°C, 24 hours) pre-weighed cheese cloth. The wheat cells were then filtered through the cloth and dried at 50°C for 24 hours before being re-weighed. The pH of the cell suspension cultures was measured using a pH meter.

4.2.16.7 Fluorescein diacetate viability assay

Wheat cells from 3 day old cultures (1.5ml) were placed in Petri-dish wells (4cm^2). These were left to acclimatise under normal growth conditions for 24 hours. Culture filtrate was then added to each well, 200µl per well and the cells left for a further 24 hours under normal growth conditions. Fluorescein diacetate was used to monitor wheat cell viability by the method of Widholm (1972). A stock solution (5mg ml^{-1} in acetone) was diluted 1:200 with SDW. 100µl of the diluted fluorescein was added to each well, and the wells left in the dark at 25°C for 10 minutes. Cells were then viewed under UV light. The intensity of fluorescence was determined by the average pixel value for each well using image analysis. Samples from each culture filtrate were replicated in 3 wells. Control wells contained un-inoculated media or 4 day old wheat cell suspension cultures which had previously been frozen at -20°C for 30 minutes. The pixel values (minus a background reading) were normalised using the arcsine transformation (arcsin$\sqrt{x}$) as the pixel value derived from the image analysis was given as a proportion of 1-100 with 1 = black and 100 = white. Differences in cell viability were analysed using single factor ANOVA and Student T-tests.

4.2.16.8 Wheat root growth inhibition

Wheat cv. Beaver seeds were germinated for 24 hours in 9cm diameter plastic Petri-dishes on Whatman no. 1 filter paper moistened with 5ml SDW. The Petri-dishes were sealed with parafilm to prevent drying out and placed at room temperature (~25 °C). Seeds with a tap root length of 5mm were selected approximately 24-36 hours after germination and 5 were placed in 5cm diameter Petri-dishes on filter paper. Culture filtrate (2.5ml) was added to the filter paper and the Petri-dishes were sealed

with Parafilm and placed at 19°C in the dark for 48 hours. Tap root length was then measured and the percentage inhibition compared to the controls determined. Each culture filtrate was added to 2 Petri-dishes of wheat roots (total 10 roots tested); control seedlings were treated with un-inoculated media or SDW.

4.3 Results

4.3.1 Colony morphology on MYG agar

Colony morphologies of the W and R-types field strains could be classed typically as having even or feathery margins thus were named 'W-morphology' and 'R-morphology' respectively (see Table 4.4, Plate 2.1). The W x R hybrids produced colonies of either W or R-morphologies. Some of the hybrids (D2/35, D2/36, D3/41, D3/48, D5/82, D5/83b and D5/100) obtained from the cross between the W-type strain 22-20 and R-type strain 23-2 displayed an intermediate colony morphology (incorporating both W and R-morphologies). The inter-specific hybrids showed either W or R- morphologies (*P. anguioides* having a W-morphology).

4.3.2 Growth rates on MYG agar

No significant differences were found between the growth rates of the W and R-type strains. However significant differences ($P<0.05$) were found between the growth rates of the W x R hybrids showing W and R-morphologies with the R-morphologies having a slower growth rate than the W-morphologies. All of the W x R hybrids were significantly ($P<0.05$) slower in growth than their parental W and R-type field strains. The W x R hybrids showing an intermediate W and R-morphology had growth rates comparable to that of the W x R hybrids with an R-morphology. The inter-specific hybrids grew significantly ($P<0.05$) slower than their R-type parent but grew at the same rate as *P. anguioides*. Fig 4.1 shows a selection of the growth rates of field strains, W x R hybrids and inter-specific hybrids showing W or R-morphologies.

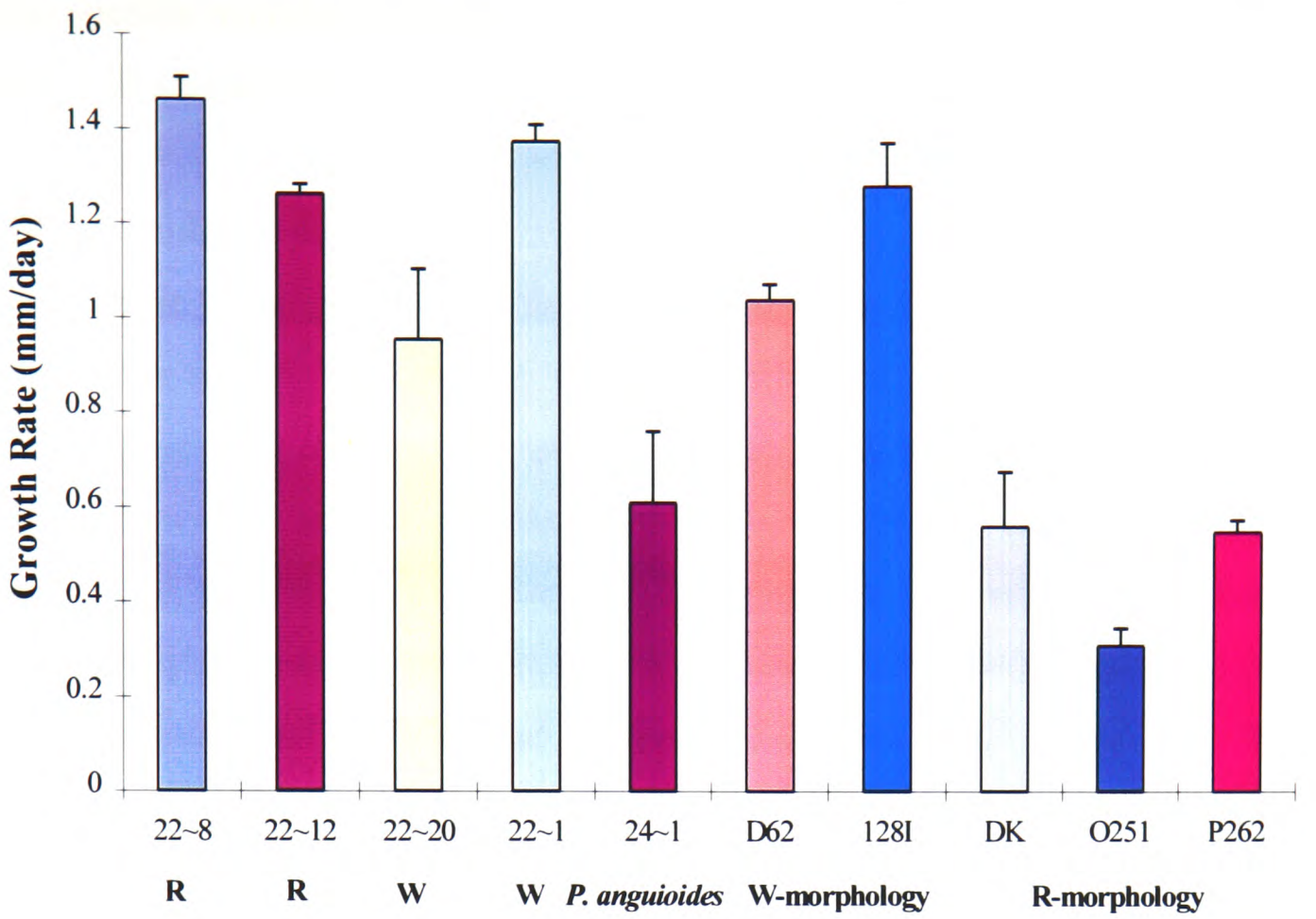


Fig 4.1: Growth rates of selected W and R-type strains and W x R- and inter-specific hybrids on MYG agar. **R**=R-type, **W**=W-type. Error bars represent the standard error for each mean.

4.3.3 Pigmentation on maize meal agar

The colour pigments of the strains and hybrids grown on maize meal agar were classed as fulvous (dark brown), olivaceous grey (green/grey), buff (reddish brown), and straw yellow (Table 4.4, Plate 4.2). The W-type strains were all olivaceous in pigmentation colour and the R-type strains all fulvous in pigmentation colour except for strain 22-119 which was olivaceous. *P. anguioides* was straw yellow in colour pigmentation. The majority of W-morphology W x R hybrids were fulvous in colour with a few olivaceous and buff pigmentations seen. The majority R-morphology W x R hybrids were also fulvous in colour pigmentation with only 2 showing an olivaceous and 1 a buff colour pigmentation. The W x R hybrids showing a combined W and R-morphology were mainly fulvous in colour pigmentation. The

inter- specific hybrids (R-type x *P. anguioides*) were largely fulvous in pigmentation with 1 olivaceous and 1 buff pigmentation seen.

4.3.4 Conidial production

Conidia were produced by all the field isolated strains and by the majority of the W x R- and inter-specific hybrids (see Table 4.4). Where no conidia were formed only hyphal growth was observed. No correlation was found between the hybrids unable to produce conidia and the parental crosses from which they came. Where conidia were formed, no differences in spore morphologies were observed within or between the W x R hybrids and parental strains. The conidia varied between straight to curved in shape, were multinucleate and contained 4-6 cross walls (Plate 5.2). Visual observations found that conidia produced by the inter-specific hybrids appeared generally longer in length and had more cross walls than conidia from field strains. W x R hybrids of *P. herpotrichoides* did not appear longer in length than the conidia produced from *P. anguioides*.

Table 4.4: Colony morphology, conidia production and molecular characterisation of the field strains and W x R and inter-specific hybrids.

Strain/hybrid	Colony morphology	Colour on maize meal	Conidia production	Product with W primers	Product with R primers
Field strains					
22-1	W	O	Yes	√	X
22-2	W	O	Yes	√	X
22-8	R	F	Yes	X	√
22-12	R	F	Yes	X	√
22-20	W	O	Yes	√	X
22-119	R	O	Yes	X	√
23-2	R	F	Yes	X	√
24-1	W	S	Yes	X	X
Inter-specific hybrids (R-type x *P. anguioides*)					
AJ	W	B	No	-	√
DQ	R	F	Yes	-	√
DK	R	O	Yes	X	√
DM	R	F	Yes	-	-
EP	R	F	Yes	-	√
FG	W	F	No	-	√
W x R hybrids					
A12	W	F	Yes	-	-
B27	R	F	Yes	-	-
C41	W	F	Yes	√	√
D62	W	F	Yes	-	√
E75	W	F	Yes	-	√
F90	W	F	Yes	-	√
G109	R	F	Yes	-	-
37I	W	F	Yes	-	√

128I	W	O	No	-	√
I131	R	O	Yes	-	-
138I	W	F	Yes	-	√
J160	W	O	Yes	-	√
J166	R	F	No	-	√
M218	R	F	Yes	√	√
O251	R	F	Yes	√	√
P262	R	F	Yes	√	√
X397	R	F	Yes	-	-
Z449a	W	B	No	-	-
Z449o	W	F	No	-	-
AE586	R	F	Yes	-	
AE588	R	F	Yes	-	√
AE591	R	F	No	√	√
D2/35	W/R	B	No	-	-
D2/36	W/R	F	Yes	-	√
D3/41	W/R	F	Yes	√	√
D3/48	W/R	O	No	-	√
D5/82	W/R	F	No	-	√
D5/83b	W/R	F	Yes	-	√
D5/100	W/R	F	No	-	-
AI382	R	B	No	-	√
AI386	R	F	No	-	√
AI387	R	F	Yes	-	-
G12/4 CH HLC	R	O	Yes	-	-
G12/4 CH MM	W	B	Yes	√	√
H3/1 FPA HLM	R	F	Yes	-	√
H1/1 FPA HLM	W	O	Yes	-	√

R	R-morphology (Slow growth/feathery margin (S/F))	O	Olivaceous	√	Positive
W	W-morphology (Fast growth/even margin (F/E))	F	Fulvous	X	Negative
		B	Buff	-	Not tested
		S	Straw yellow		

4.3.5 Molecular characterisation

The non specific primers ITS4 and ITS5 were used identify the ITS region of the nuclear ribosomal DNA. The ITS primers generated a single band of approximately 0.6kb (see Figure 4.2) from all the selected W and R-types and W x R- and inter-specific hybrids tested. This size corresponded to the expected size according to the ITS of other fungi (White *et al.*, 1990).

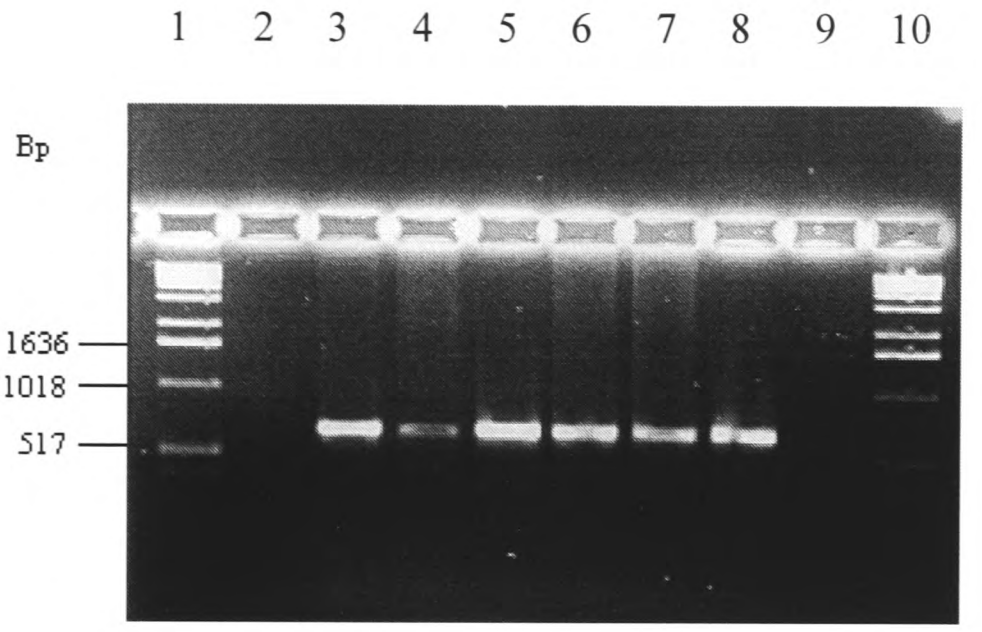


Fig 4.2: Specific PCR amplification of W and R-types and inter specific and W x R hybrids with primers ITS4 and ITS5. Lanes 1 and 10 DNA 1.22kb ladder molecular weight marker, lanes 3-8 strains and hybrids (3: 22-20, 4: 22-8, 5: DK, 6: C41, 7: O251, 8: P262), lanes 2 and 9 negative controls (no DNA template).

The restriction digest using the enzyme *Hae III* recognises the sequence GG/CC and generates fragments with blunt ends. Two fragments were yielded from this enzyme digest, a fragment common to both W and R-types and a second fragment which was smaller in size from the R-types compared to the W-types (see Figure 4.3).

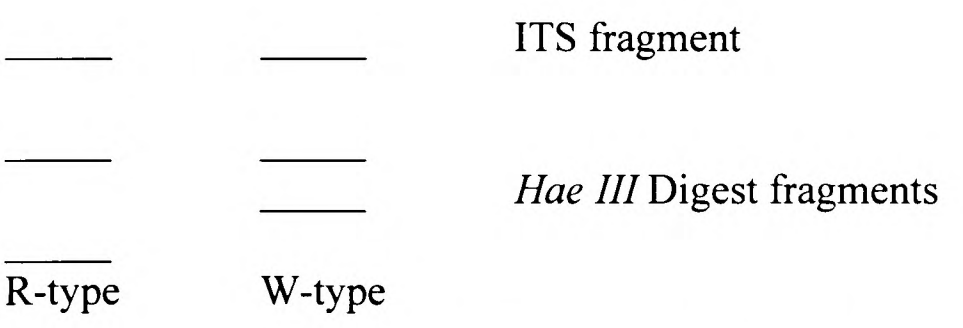


Fig 4.3: Typical banding patterns obtained from a *Hae II* digest of the PCR amplified ITS fragments of R and W-type strains.

The W and R-type strains digested ITS PCR products showed corresponding W and R-type banding patterns. The W x R hybrids tested C41, P262 and O251 showed banding patterns of an R-type and the inter-specific hybrid tested DK showed a banding pattern also of an R-type. Only selected hybrids were tested due to time restrictions and resources available.

The primer pairs Ty16F/R and TaO5F/R were used for a specific PCR to identify W and R-types respectively. The primer pair TY16F/R gave a product of approximately 1.05kb and TAO5F/R a product of 0.33kb. Following PCR amplification with these primer pairs, the field (wild-type) strains showed amplification of either the W or R PCR fragment, which confirmed their previous pathotype designation of W or R-type on colony morphology (see Table 4.4) and *Hae III* enzyme digest. Selected hybrids were also tested with both primer pairs. Where tested, all W x R hybrids gave amplification products with both primer systems (see Table 4.4 and Figure 4.4).

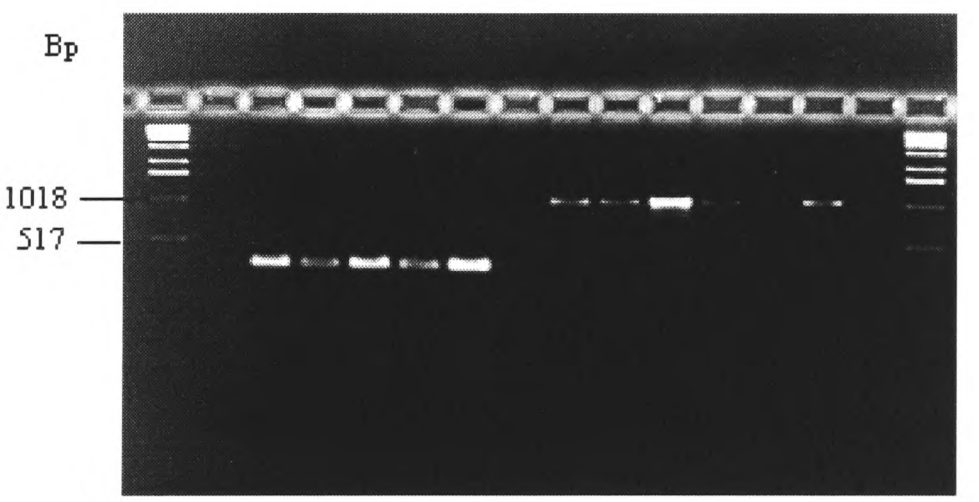


Fig 4.4: Specific amplification of strains and hybrids with primer pairs Ty16F/R and TaO5F/R. Lanes1 and 16 DNA 1.22kb ladder molecular weight marker, lanes 3-8 primer pair TaO5F/R (3: P262, 4: O251, 5: C41, 6: DK, 7: 22-8, 8: 22-20), lanes 9-14 primer pair Ty16F/R (9: P262, 10: O251, 11: C41, 12: DK, 13: 22-8, 14: 22-20), lanes 2 and 15 negative controls (no DNA template).

4.3.6 Pathogenicity assessment

The pathogenicity to wheat, barley and rye of the parental strains and hybrids was determined by a visual assessment of the depth of penetration through the stem base of the lesions and by a microscopic assessment for the presence of infection plaques. A strain was determined to be pathogenic if at least one plant from a pot was given a disease infection score greater than 0 (i.e. the coleoptile at least was infected). Strains displaying high pathogenicity penetrated through 4-5 leaf sheaths giving a mean disease infection score of between 9 and 11. The disease scores were generally consistent between the replicate plants in a pot.

A blocking effect was seen to occur between the 3 blocks and the 3 hosts (wheat, barley and rye) but there were no interactions. This blocking effect may be attributed to the experiment being performed in an end glasshouse unit thus having 2 external sides and hence the blocks at these edges received more sunlight which may have lead to an uneven temperature distribution. One block had host plants a week younger than the other blocks due to the original plants being eaten by mice. This may have altered their susceptibility to infection. There could have also been an uneven distribution of inoculum around the plants and competition from other fungi such as *Fusarium* which was sometimes present. These latter blocking effects appeared random.

The W and R-type strains were pathogenic to wheat, barley and rye indicating that conditions were suitable for infection. From the mean disease infection scores generally the W-type strains were of higher pathogenicity to wheat than the R type strains. The R-type strains were of higher pathogenicity to barley than the W-types. The W -type strains were slightly more pathogenic to rye than the R-type strains. The hybrid progeny varied in their pathogenicity on the three host species, some being more pathogenic and others less pathogenic than the respective parental strains with several being non-pathogenic (no disease symptoms).

Strains and hybrids that were pathogenic to any of the hosts were divided into 2 classes, low and high pathogenicity. Strains/hybrids showing low pathogenicity were classified as having the sum disease infection score less than 5 (24 plants from 3 pots). Strains classed as having high pathogenicity had a sum disease infection score greater than 5.

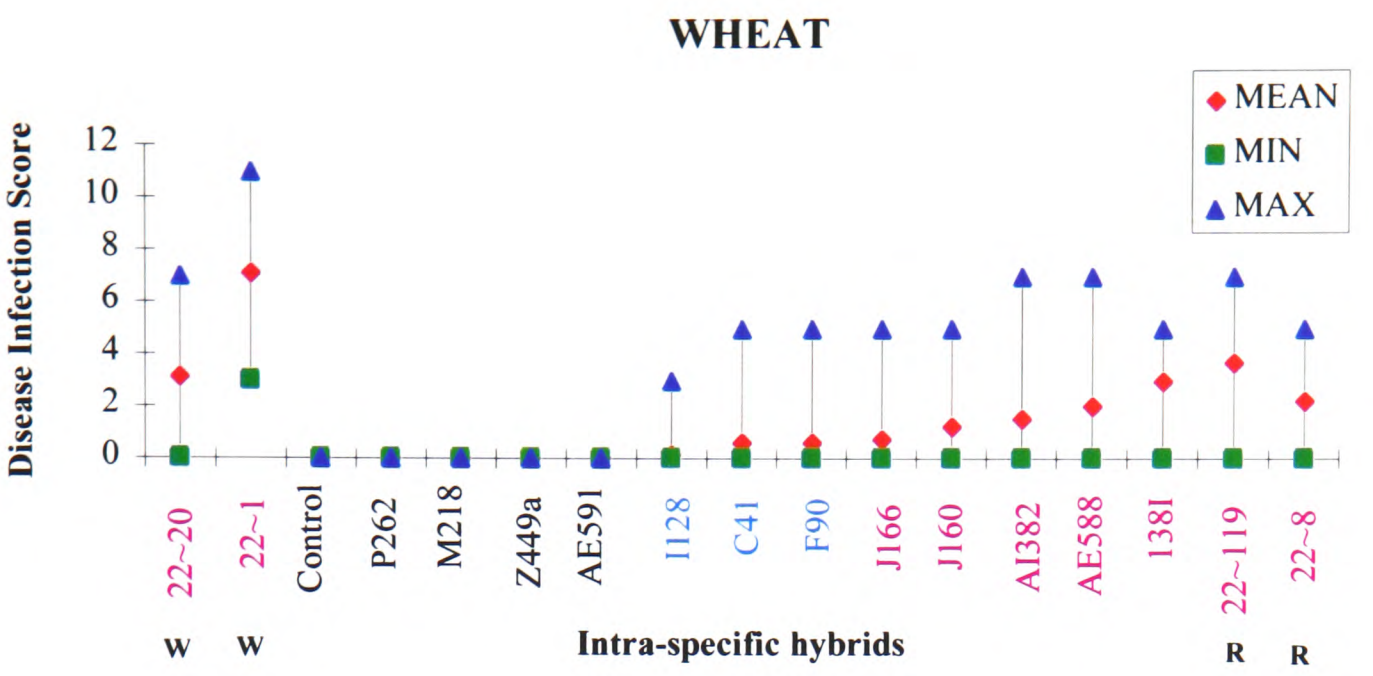
Fig 4.5 shows the mean, minimum and maximum disease infection scores (untransformed data) of the parental strains W-type strains 22-20 and 22~1 and R-types 22-8 and 22~119 and selected hybrid progeny. It can be seen that the W-type strains 22-20 and 22~1 were more pathogenic to wheat and rye than the R-type 22-8 and 22~119. The R-type strains were more pathogenic to barley than the W-type strains. The hybrids M218, Z449a, AE591, I128 and F90 were either non pathogenic or of low pathogenicity to wheat, barley and rye. There was no correlation between the hosts to which the strains showed low or non-pathogenicity. The hybrids P262 and C41 were of high pathogenicity to rye but of low or non-pathogenic to wheat and barley. The hybrids J160 and I138 were of high pathogenicity on wheat and barley but of low pathogenicity on rye. The hybrids AI382 and AE588 were of high pathogenicity to wheat but non-pathogenic to barley and rye. The hybrid J166 was of high pathogenicity to wheat and of low pathogenicity to barley and rye. The pathogenicity of the W x R hybrids was generally less then the pathogenicity of the parental strains on the respective hosts.

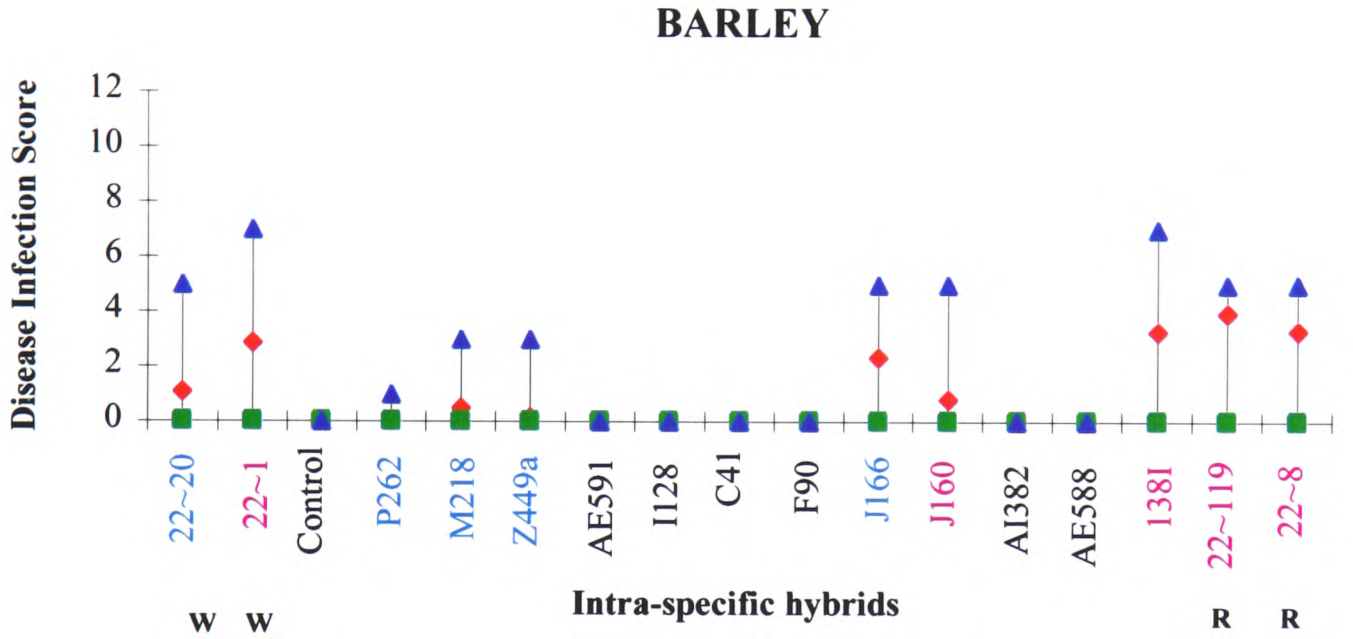
Fig 4.6 shows the mean, minimum and maximum disease infection scores (untransformed data) of the parental strains R-type 22-12 and *P. anguioides* and selected inter-specific hybrids inoculated on to wheat, barley and rye. The R-type strain 22~12 was more pathogenic to wheat than barley or rye. *P. anguioides* was of low pathogenicity to wheat, barley and rye. The inter-specific hybrids showed varying levels of pathogenicity. The hybrids EP and FG were of low pathogenicity or non-pathogenic to wheat, barley and rye. In contrast hybrids AJ, DM, DQ and DK showed high levels of pathogenicity to wheat, barley and rye with hybrids DQ and

DK showing higher levels of pathogenicity than that observed with the parental strain 22-12.

The pathogenic inter-specific hybrids were generally higher in pathogenicity than the pathogenic W x R hybrids on the three hosts.

Analysis of the total data set which included all strains and hybrids on wheat, barley and rye using the Kruskal-Wallis test found significant differences ($P<0.01$) within the total data set. Significant differences ($P<0.01$) were also found when the un-inoculated controls were removed and when only strains and hybrids deemed as pathogenic were analysed. The data sets were then analysed separately for wheat, barely and rye. These results showed that significant differences ($P<0.01$) occurred between the strains and hybrids when the un-inoculated controls were removed and also between the strains and hybrids deemed as pathogenic on the respective hosts.





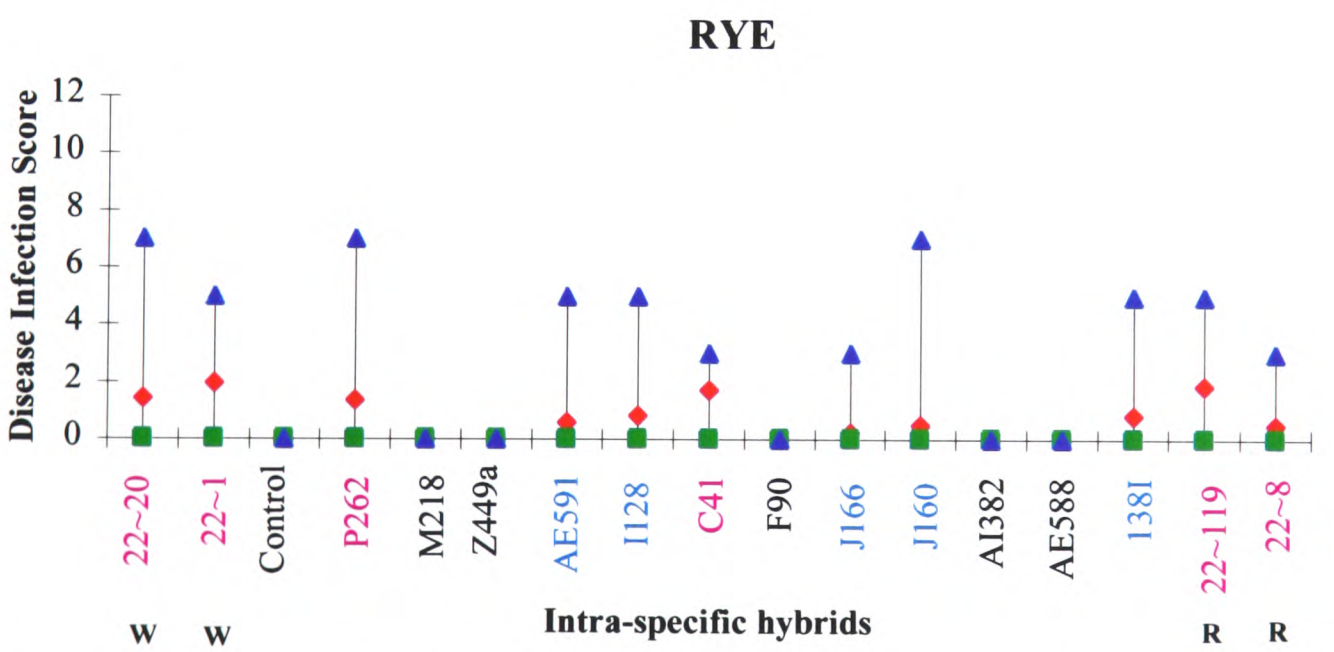


Fig 4.5: Graphs showing the means and range of disease infection scores (untransformed data) of strains and W x R hybrids inoculated on to wheat, barley and rye. **R**=R-type, **W**=W-type.
High, low and non-pathogenic strains and hybrids on respective cereal hosts.

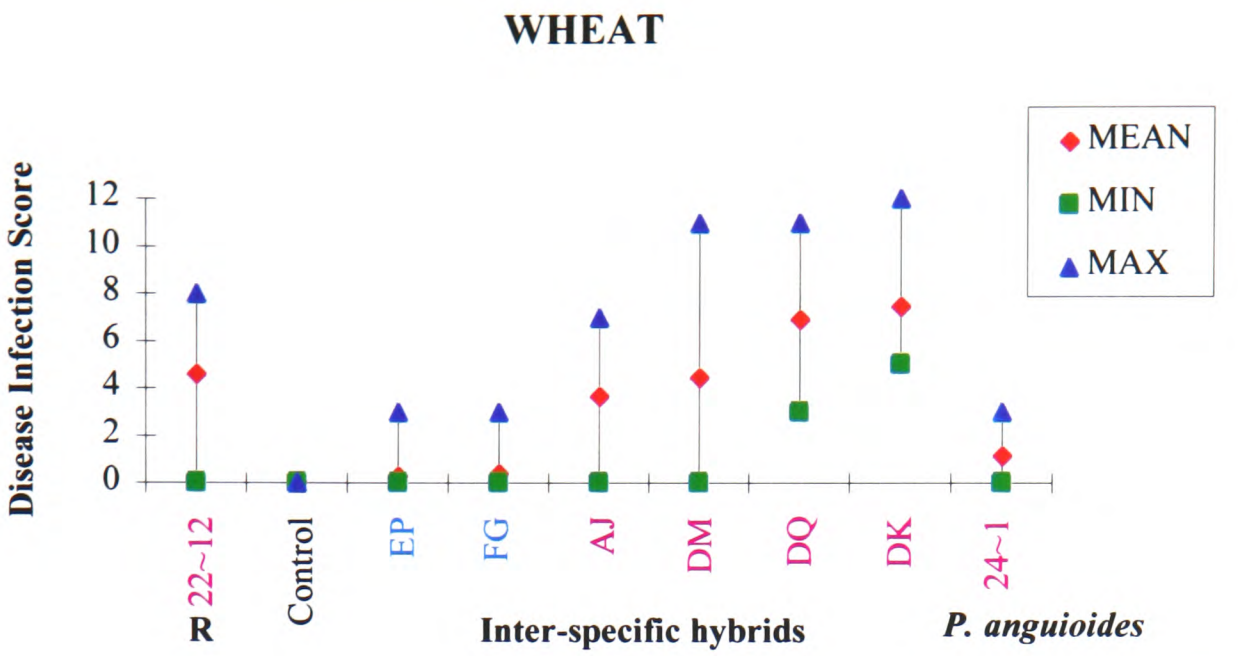


Fig 4.6: Graphs showing the means and range of disease infection scores (untransformed data) of strains and inter-specific hybrids inoculated on to wheat, barley and rye. **R**=R-type.
High, low and non-pathogenic strains and hybrids on respective cereal hosts.

4.3.7 Microscopic examination

A microscopic examination was made for the presence of infection plaques. This allowed for the identification of hybrids which were asymptomatic (unable to cause disease symptoms but can still colonise the host). This analysis also shows the relationship between the disease symptoms and the infection plaques of this fungus at a time point 8 weeks after inoculation. The first, second and third leaf sheaths were examined for infection plaques which were of a W or R-type. The W-types plaques were composed of swollen hyphal cells loosely associated, whereas the R-type plaques were more discrete and circular and composed of closely associated cells (Plates 5.2, 5.3). Infection plaques varied in size and in some cases several W-type plaques merged to form one very large plaque.

Figures 4.7 and 4.8 show the distribution of infection plaques on the first, second and third leaf sheaths of selected strains and hybrids. The number of infection plaques are represented by the mean infection plaques score with a score of 1 indicating that 1-25 infection plaques were seen and through to a score of 5 indicating that over 100 infection plaques were seen. Fig 4.7 shows the W-type strain 22-1, R-type strain 22-12 and an inter-specific hybrid DK. Infection plaques were seen on the 1^{st}, 2^{nd} and 3^{rd} leaf sheaths with the greatest number on the 1^{st} leaf sheath and least number on the 3^{rd}. These strains and inter-specific hybrids represent typical strains and hybrids classed as having high pathogenicity to wheat, barley and rye. The W x R hybrid J160 is classed as having high pathogenicity to wheat and barley and low pathogenicity to rye. Infection plaques from hybrid J160 were only seen on the 1^{st} and 2^{nd} leaf sheath with numbers being much lower compared to the highly pathogenic strains and hybrids on all three hosts. Although classed as having low pathogenicity to rye infection plaque numbers produced by hybrid J160 were comparable to those seen on wheat and were greater than those seen on barley.

Fig 4.8 shows the distribution of infection plaques of the W x R hybrids M218, AI382, P262 and I128. The W x R hybrid M218 produced infection plaques only on

barley where from the disease symptoms it is classed as being of low pathogenicity on barley. No infection plaques were found on wheat or rye where from disease symptoms it is classed as being non-pathogenic. The W x R-hybrids AI382, P262 and I128 are classified as being asymptomatic on barley, wheat and rye respectively thus no disease symptoms were seen on these hosts but infection plaques were detected. From the disease symptoms the hybrid AI382 is non-pathogenic on barley and rye and of high pathogenicity to wheat. This corresponds with the infection plaques found on the 1st leaf sheath on wheat and on the 1st and 2nd leaf sheaths on barley. The hybrid P262 produced infection plaques on the 1st, 2nd and 3rd leaf sheaths of wheat although no disease symptoms were seen on this host. Infection plaques were seen on the 1st and 2nd leaf sheaths on barley and rye where it is classed as being of low and high pathogenicity respectively. The hybrid I128 produced infection plaques on the 1st, 2nd and 3rd leaf sheaths of barley. However no disease symptoms were seen on this host. Infection plaques were seen in lower numbers on wheat and rye compared to barley where it is classed as being of low pathogenicity.

The hybrids which showed low levels of pathogenicity or were non-pathogenic to a host species produced infection plaques in much lower numbers and were not always seen to colonise to the 3rd leaf sheath compared to the highly pathogenic strains and hybrids on all 3 host species. No infection plaques were seen on the control plants.

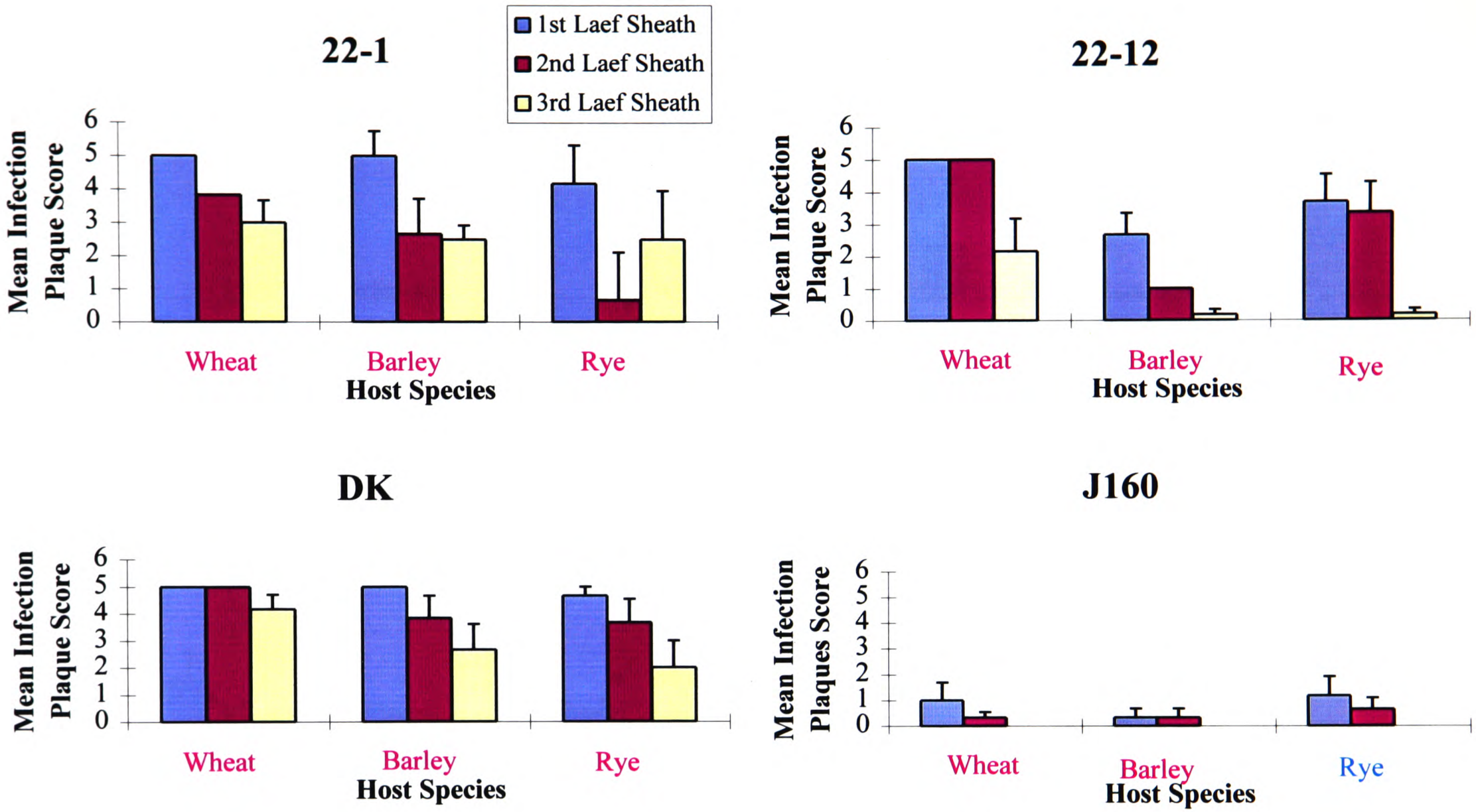


Fig 4.7: Graphs showing the distribution of infection plaques on the leaf sheaths of wheat, barley and rye of the W-type 22-20, R-type 22-12, W x R hybrid J160 and inter-specific hybrid DK. Error bars represent the standard error of the mean. High pathogenicity, low pathogenicity or non-pathogenicity to cereal hosts wheat, barley and rye.

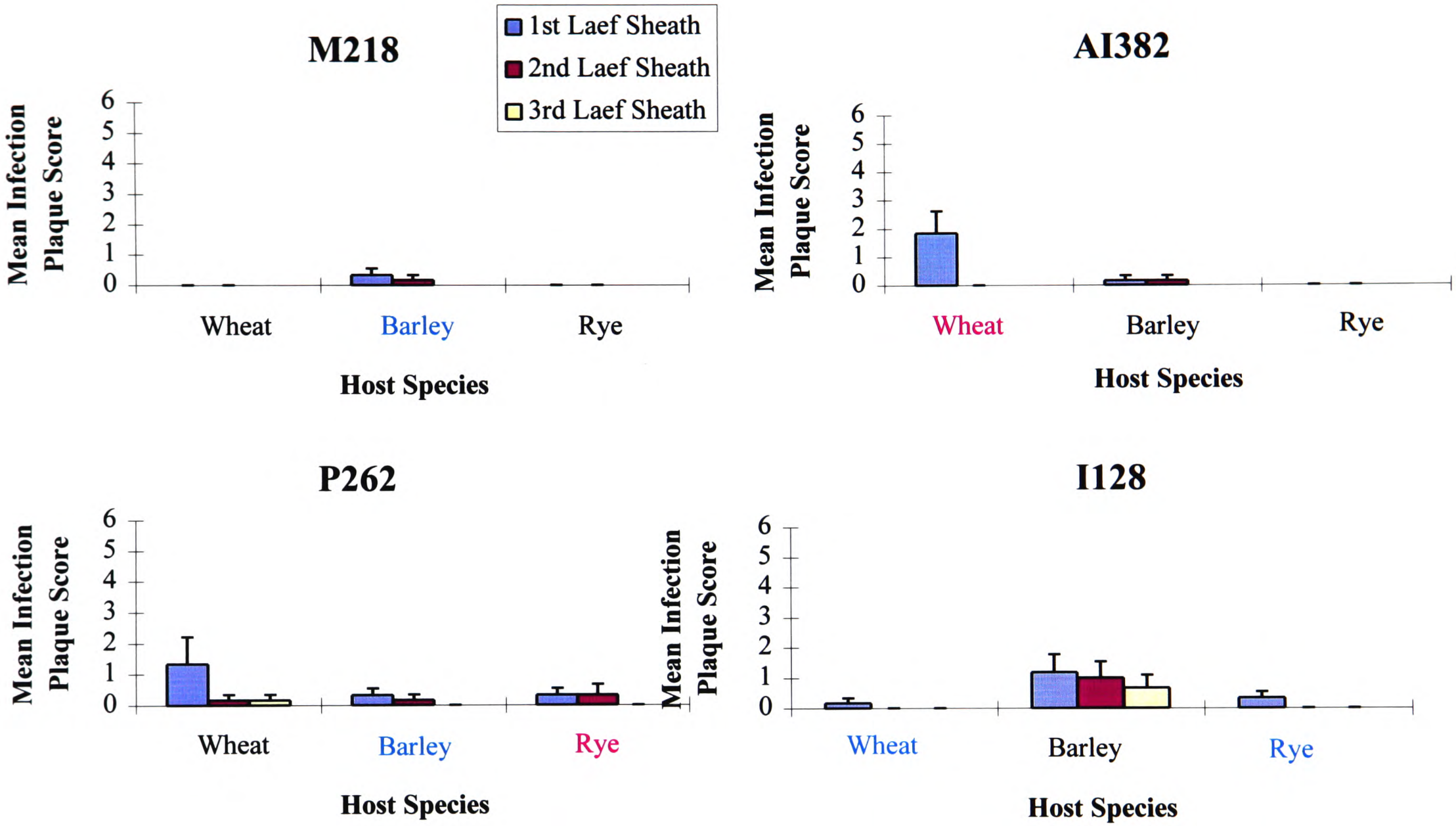


Fig 4.8: Graphs showing the distribution of infection plaques on the leaf sheaths of wheat, barley and rye of the W x R hybrids M218, AI382, P262 and I128. Error bars represent the standard error of the mean. High pathogenicity, low pathogenicity or non-pathogenicity to cereal hosts wheat, barley and rye.

The total number of infection plaques was greater on wheat compared to barley and rye and this can be seen in Figure 4.9 which shows the sum of the infection plaques score data of selected strains and hybrids. The number of infection plaques were similar on the 1st leaf sheath on the three hosts but on barley and rye fewer infection plaques were found on the 2nd and 3rd leaf sheaths compared to wheat.

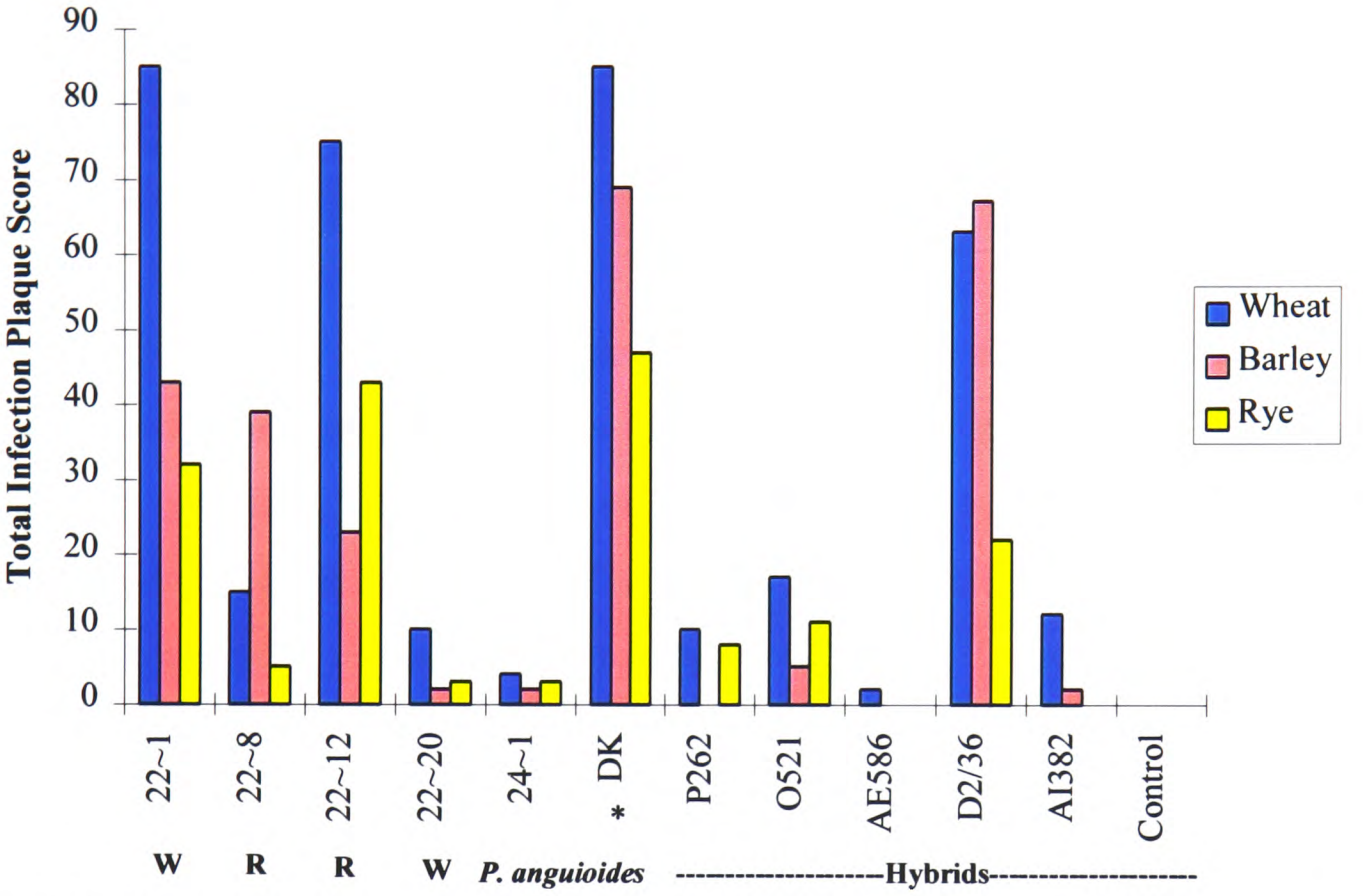


Fig 4.9: Total infection plaque scores of selected strains and hybrids on wheat, barley and rye. **R** = R-type, **W** = W-type, **Hybrids** = W x R and inter-specific hybrids, * = hybrid R x *P. anguioides*.

Table 4.5 is a summary of the classes of pathogenicity into which the strains and hybrids could be allocated on wheat, barley and rye. This summary is based upon the ability of the strains and hybrids to cause disease symptoms and the ability to produce infection plaques in the absence of disease symptoms. Strains and hybrids classed as having high pathogenicity had a sum disease infection score greater than 5, strains and hybrids classed as having low pathogenicity had a sum disease infection score less than 5. Hybrids classed being asymptomatic produced infection plaques on examined leaf sheaths but no disease symptoms were seen. Hybrids

classed as being non pathogenic produced neither disease symptoms or infection plaques.

Table 4.5: A summary of the numbers of pathogenic, non pathogenic and asymptomatic strains/hybrids occurring on wheat, barley and rye.

Plant Species	Number of strains/hybrids showing high pathogenicity	Number of strains/hybrids showing low pathogenicity	Number of non-pathogenic hybrids	Number of asymptomatic hybrids
Wheat	26	18	2	4
Barley	22	20	6	2
Rye	16	22	11	1

Generally the hybrids found to be asymptomatic on wheat, barley or rye were found to be of low pathogenicity to other host species. The hybrids that were non-pathogenic on wheat, barley or rye were also found to be of low or to be non-pathogenic to the other host species. The W x R hybrids showing high pathogenicity on wheat were either of high or low pathogenicity to rye. Those W x R hybrids of high pathogenicity to rye were of high or low pathogenicity to wheat. The W x R hybrids of high pathogenicity to wheat or rye varied in their pathogenicity to barley.

4.3.8 Infection plaque assay

The ability of the strains and hybrids to produce infection plaques was determined *in vitro.* The preliminary studies showed that full strength MYG agar sloped on to a glass Petri-dish induced infection plaque formation the best (see Plate 4.3 and Appendix 4.2). This method also allowed for radial growth of the strains and hybrids showing their viability. Infection plaques were characterised as aggregated swollen cells forming from hyphae (Plate 4.3). These infection plaques generally appeared on the bottom of the Petri-dish within a short distance of the edge of the agar slope. Hyphal growth was seen to extend further out across the bottom of the Petri-dish: however infection plaques were rarely seen this distance away from the agar. Significant differences ($P<0.05$) were found between the numbers of infection plaques produced by the strains and hybrids (the data was transformed using

Cochran's square root transformation for small numbers $\sqrt{(X+0.5)}$). Fig 4.10 shows the mean number of infection plaques produced by some of the strains and hybrids. The numbers of infection plaques were counted over 10 graticules just after the edge of the sloped MYG agar. The W and R-type strains all produced infection plaques with the R-type strains producing significantly more plaques ($P<0.05$) than the W-type strains. The W x R- and inter-specific hybrids produced varying numbers of infection plaques with many unable to produce infection plaques under the test conditions. Of those hybrids producing infection plaques, the number of plaques formed was generally less than their respective parental strains, with the exception of the inter-specific hybrid DM which produced more than either parental R-type strain 22-12 and *P. anguioides* strain 24-1.

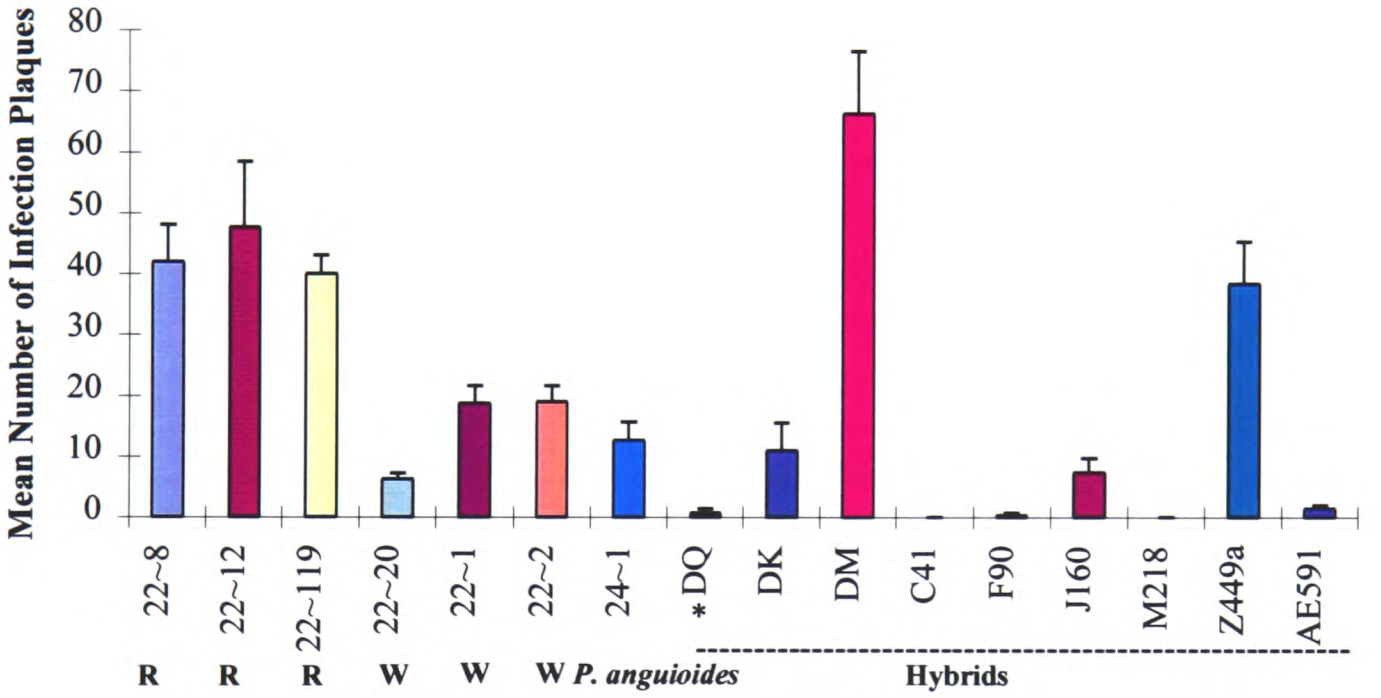


Fig 4.10: The mean number of infection plaques produced *in vitro* by some of the strains and hybrids on the bottom of glass Petri-dishes. **R**=R-type, **W**=W-type, **Hybrids** = W x R hybrids, * = hybrid R x *P. anguioides*. Error bars represent the standard error for each mean.

Application of the Tukey Test for multiple comparisons indicated overlying sets of similarity in the numbers of infection plaques produced by the strains and hybrids. This can be seen in Table 4.6 with the data arranged in decreasing numbers of infection plaques produced.

Table 4.6: Results of the Tukey Test for multiple comparisons of the number of infection plaques produced by the strains and hybrids *in vitro*. Strains/hybrids listed in decreasing numbers of infection plaques produced. Strains that share the same letter are not significantly ($P \geq 0.05$) different.

Strain/Hybrid	Description	Mean Number of Infection Plaques						
DM	Inter-specific hybrid	67	a					
22-12	R-type	48	a	b				
22-8	R-type	42		b				
22-119	R-type	40		b	c			
Z449a	W x R hybrid	38		b	c			
FG	Inter-specific hybrid	27		b	c	d	h	
23-2	R-type	76		b	c	d	h	
D62	W x R hybrid	19			c	d	h	i
22-2	W-type	19			c	d	h	i
22-1	W-type	19			c	d	h	i
24-1	*P. anguioides*	13				d	h	i
G12/4 CH HLC	W x R hybrid	11				d	h	i
DK	Inter-specific hybrid	11					h	i
EP	Inter-specific hybrid	8					h	i
H3/1 FPA HLM	W x R hybrid	7					h	i
J160	W x R hybrid	7					h	i
Z449o	W x R hybrid	7					h	i
22-20	W-type	6					h	i
D3/48	W x R hybrid	6					h	i
G12/4 CH MM	W x R hybrid	6					h	i
D3/41	W x R hybrid	4					h	i
128I	W x R hybrid	3						i
F84	W x R hybrid	2						i
AI386	W x R hybrid	2						i
D2/36	W x R hybrid	2						i
G109	W x R hybrid	2						i
AE591	W x R hybrid	1						i
B22	W x R hybrid	1						i
AJ	Inter-specific hybrid	1						i
138I	W x R hybrid	1						i
DQ	Inter-specific hybrid	1						i
F90	W x R hybrid	0						i
H1/1 FPA HLM	W x R hybrid	0						i
AI387	W x R hybrid	0						i
AI382	W x R hybrid	0						i
D5/100	W x R hybrid	0						i

D5/82	W x R hybrid	0						i
D2/35	W x R hybrid	0						i
AE588	W x R hybrid	0						i
AE586	W x R hybrid	0						i
X397	W x R hybrid	0						i
P262	W x R hybrid	0						i
O251	W x R hybrid	0						i
M218	W x R hybrid	0						i
J166	W x R hybrid	0						i
I131	W x R hybrid	0						i
37I	W x R hybrid	0						i
E75	W x R hybrid	0						i
C41	W x R hybrid	0						i
A12	W x R hybrid	0						i

Infection plaque size was determined in relation to the size of a graticule (0.25 x 0.25mm), with a score of 1 equalling the size of a single graticule square. Significant differences ($P<0.05$) were found between the sizes of the infection plaques both between and within the strains and hybrids. This could not be related to either the W or R-type strains nor to the W x R or inter-specific hybrids. No differences in morphology of the infection plaques could be detected between W and R-types or between any of the hybrids. Fig 4.11 shows the plaque size of selected strains and hybrids that produced infection plaques. It can be seen that infection plaque size of the hybrids varied but the majority were smaller in size compared to the parental strains.

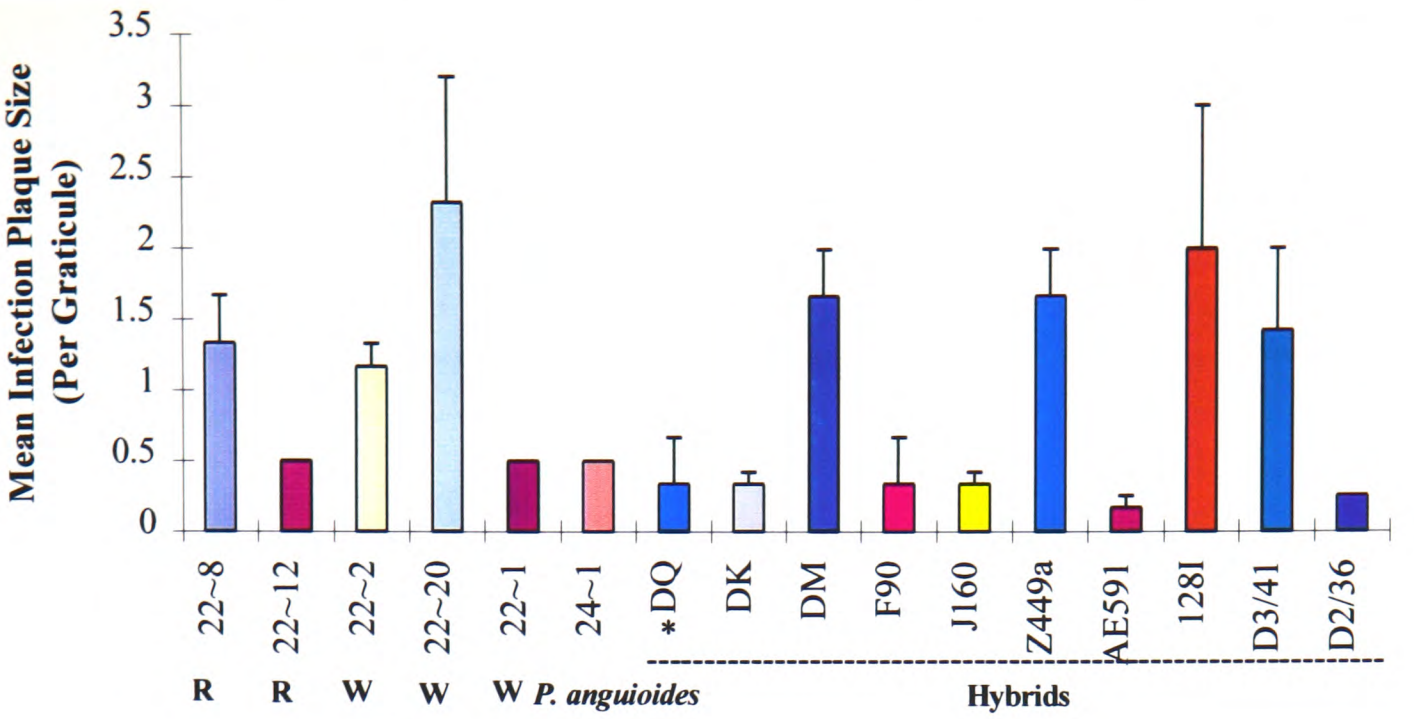


Fig 4.11: The mean size of infection plaques produced *in vitro* by some of the strains and hybrids on the bottom of glass Petri-dishes. 1 = a single graticule size. **R** = R-type, **W** = W-type, **Hybrids** = W x R hybrids, * = hybrid R x *P. anguioides*. Error bars represent the standard error for each mean.

No significant correlations were found between 1) the average size of the infection plaques and the number of infection plaques, 2) between the size of the infection plaques and the growth rate of the strain/hybrid or 3) between the growth rate of the strain/hybrid and the number of infection plaques produced. Growth rates of the strains and hybrids were found to be similar to those described in Section 4.3.2.

4.3.9 Detection of secondary metabolites

Preliminary studies characterised the wheat cell suspension cultures. An increase in fresh and dry weight was seen over the 10 days of growth. Figure 4.12 shows the growth curves obtained from measuring both fresh and dry weight. Both graphs show a typical lag phase until day 4; this can be attributed to the cells adapting to the medium. An exponential phase was seen then until day 5 and this was followed by a stationary/ death phase until day 10.

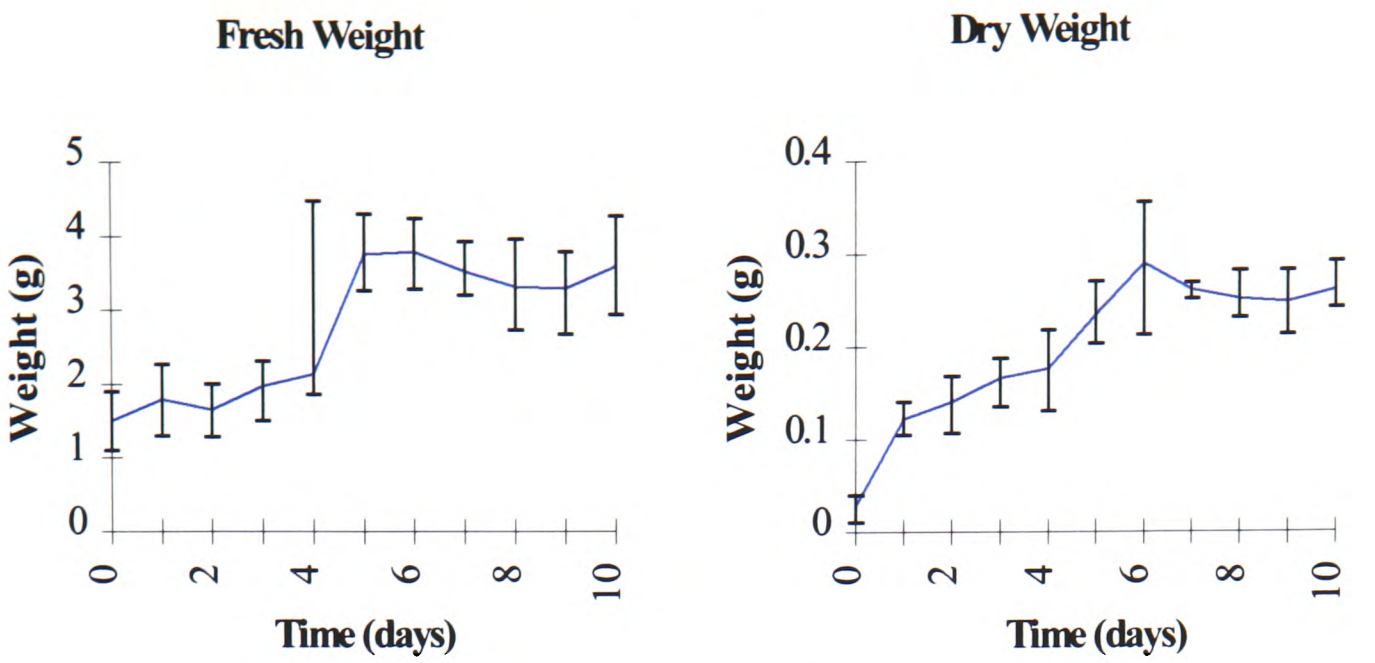


Fig 4.12: The mean fresh and dry weights of a wheat cell suspension cultures measured over time. Error bars show the range of measurements found for each point.

Although similar growth patterns were detected using fresh and dry weights, dry weight measurement are more accurate as fresh weight includes the water content of the cells and surface wetting which leads to increased variability.

From these data it was decided that using the wheat cells 4-5 days after sub-culturing would be most reliable as this utilised the cells in the exponential phase of growth.

The pH of the wheat cell suspension cultures decreased from an initial pH of 5.8 (without the addition of fungal culture filtrates) to pH of 4.1 after 5 days growth of wheat cells, until the 10^{th} day final assessment the pH of the cultures remained at about pH 4.1. An initial decrease in pH from pH 5.8 to pH 5.68 was seen after the media was autoclaved. Addition of 3ml of an old wheat cell culture then caused the pH to drop to 5.3. The decrease in pH then from 5.3 to 4.1 during cell growth can be attributed to metabolic processes; the preferential uptake of ammonia at high concentrations and then the uptake of nitrates. The stabilisation of pH which was seen after 5 days may be attributed to the cessation of ammonia and nitrate uptake, as the growth requirements then probably were supplied by the stored intra-cellular nitrates.

4.3.10 Fluorescein diacetate viability assay using MYG fungal culture filtrates

Viable plant cells are able to take up fluorescein diacetate and cleave the molecule to release fluorescein which, on excitation with UV light yields a greenish yellow fluorescence. Non-viable cells do not do this. Wheat cell viability was expressed as a pixel value and the data was transformed using the arcsine transformation before being analysed using a single factor ANOVA. A loss in fluorescence compared to the control wheat cells was taken to indicate a loss in wheat cell viability. A variation in control media pixel values was seen due to the wheat cells suspension cultures being sub-cultured and tested with fungal culture filtrates on a weekly basis. It was not possible to compare pixel values between sample days. Samples of culture filtrate from strain 22-8 and inter-specific hybrid DK reduced wheat cell viability as determined using this assay method. Significant reductions ($P<0.01$) in wheat cell viability obtained with filtrates of fungal cultures from strain 22-8 and hybrid DK which had been growing for at least 15 days (see Table 4.7). Significant differences ($P<0.01$) were also found between strain 22-8 and hybrid DK culture filtrates in their ability to reduce wheat cell viability; these differed for each sampling time (see Plate 4.4). Fig 4.13 shows the reduced fluorescence of wheat cells by the addition of fungal culture filtrates obtained from strain 22-8 and DK obtained after 21 days growth.

Table 4.7: The reduction in wheat cell viability by MYG media culture filtrates increasing in age from strain 22-8 and hybrid DK sampling every 3 days over a 30 day period. Transformed average pixel value presented.

Age of fungal culture filtrate (days)	Strain 22-8 Pixel Value	Inter-specific hybrid DK Pixel Value	Control Media Pixel Value
3	12.97	12.47	13.55
6	20.21	13.99	18.41
9	13.49	13.57	11.46
12	14.32	19.7	21.35
15	26.19	19.11	27.62
18	6.7 *	6.9 *	13.39
21	12.4 *	8.8 *	18.58
24	16.33 *	12.68 *	25.53
27	9.26 *	15.3 *	26
30	15.13 *	8.67 *	23.07

* Significant reduction ($P<0.01$) in wheat cell viability compared to control wheat cells.

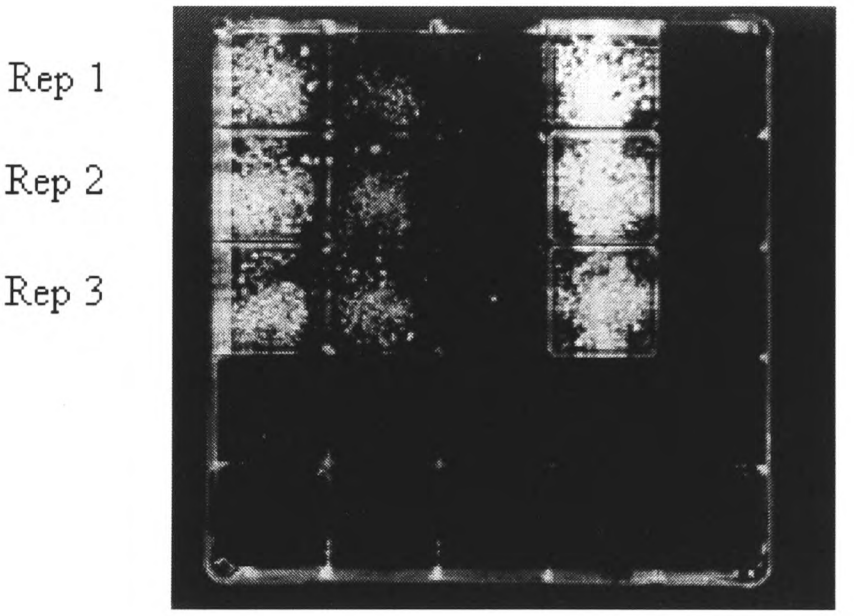


Figure 4.13: The reduced fluorescence of wheat cells by the addition of fungal culture filtrates obtained from strain 22-8 and hybrid DK obtained after 21 days growth.

As initial studies using strain 22-8 and hybrid DK found that only fungal culture filtrates grown for a minimum of 18 days decreased wheat cell viability, further

studies were only carried out on filtrates after 18 days growth to save on time and resources. Studies using culture filtrates 18, 21, 24, 27 and 30 days old from strain 22-20 did not significantly reduce wheat cell viability. In contrast the culture filtrates from the W x R hybrids P262, O251 and C41 significantly reduced ($P<0.01$) wheat cell viability at most of the sample times (see Tables 4.8 and 4.9).

Table 4.8: The reduction in wheat cell viability by MYG media culture filtrates increasing in age from strain 22-20 and hybrid C41 sampling only after 18 days growth of a 30 day period. Transformed average pixel value presented.

Age of fungal culture filtrate (days)	Strain 22-20 Pixel Value	W x R hybrid C41 Pixel Value	Control Media Pixel Value
18	4.41	0.604 *	5.43
21	7.69	0.854 *	4.83
24	13.48	7.92	9.59
27	6.11	0 *	8.84
30	7.91	0 *	3.85

* Significant reduction ($P<0.01$) in wheat cell viability compared to control wheat cells.

Table 4.9: The reduction in wheat cell viability by MYG media culture filtrates increasing in age from hybrids P262 and O251 sampling only after 18 days growth of a 30 day period. Transformed average pixel value presented.

Age of fungal culture filtrate (days)	W x R hybrid P262 Pixel Value	W x R hybrid O251 Pixel Value	Control media Pixel Value
18	4.51 *	3.46 *	11.25
21	1.45 *	0 *	4.69
24	1.81 *	1.208 *	4.76
27	0.854 *	1.45*	5.87
30	5.08 *	0.854 *	9.09

* Significant reduction ($P<0.01$) in wheat cell viability compared to control wheat cells.

The pH of the fungal culture filtrates rose from 6.1 to approximately 8.2 over the 30 days growth for both strain 22-8 and hybrid DK. Fungal pigments were seen to be secreted into the media after about 12 days growth from strain 22-8 and hybrids DK,

P262, O251 and C41 the intensity of which varied both between and within strain and hybrid culture filtrates, and usually increased daily. Strain 22-20 secreted very few pigments into the culture medium.

Microscopic examination of selected wheat cells treated with fungal culture filtrates indicated that the fungal pigments were present in their vacuoles.

4.3.11 Wheat root growth inhibition using MYG fungal culture filtrates

No root growth inhibition was detected when culture filtrates from strain 22-8 or hybrid DK over the time course of fungal growth were added to wheat roots. Significant increases in root growth ($P<0.05$) were seen between the addition of SDW and MYG media to the roots. The roots treated with SDW increased on average by 10.8 ± 4.7mm whereas roots treated with MYG increased on average by 21.4 ± 2mm. The presence of malt, yeast and glucose in the MYG media may be providing extra nutrients and energy thus increasing root growth compared to the addition of SDW. Significant ($P<0.05$) increases in root growth were seen with some of the culture filtrates from strain 22-8 and hybrid DK, these were comparable to those seen when MYG media alone was added to the roots and appeared to occur randomly with the fungal culture filtrate samples, see Table 4.10.

Table 4.10: The effect of MYG media fungal culture filtrates grown over a time course from strain 22-8 and hybrid DK on the average wheat root length.

Age of fungal culture filtrate (days)	Strain 22-8 Wheat root length increase expressed as % of MYG media controls	Inter-specific hybrid DK Wheat root length increase expressed as % of MYG media controls
3	133.41 *	142.6 *
6	99.86	94.37
9	99.97	103.97
12	215.56 *	187.86*
15	148.39	176.53
18	115.87	95.33
21	123.8 *	153 *
24	92.95	91.73
27	119.67	124.59
30	101.47	116.88

* Significant ($P<0.05$) increase in wheat root length compared to MYG media controls.

Significant increases ($P<0.05$) in root growth were also seen with some of the culture filtrates from P262, O251 and C41. However there were some significant reductions ($P<0.05$) in root length when treated with 21 and 24 day old culture filtrates from strain 22-20, see Table 4.11.

Table 4.11: The effect of MYG media fungal culture filtrates grown over a time course from strain 22-20 and W x R hybrids P262, O251 and C41 on the average wheat root length.

Age of fungal culture filtrate (days)	Strain 22-20 Wheat root length increase expressed as % of MYG media controls	W x R hybrid P262 Wheat root length increase expressed as % of MYG media controls	W x R hybrid O251 Wheat root length increase expressed as % of MYG media controls	W x R hybrid C41 Wheat root length increase expressed as % of MYG media controls
18	72.92	152.79	139.58	97.75
21	66.66 ^	127.76 *	95.53	93.19
24	85.34 ^	139.84 *	117.63 *	97.17
27	67.31	112.37	111	99.95
30	132.97	104.78	90.29	153.74

^ Significant ($P<0.05$) reduction in wheat root length compared to MYG media controls.

* Significant ($P<0.05$) increase in wheat root length compared to MYG media controls.

4.3.12 Fluorescein diacetate viability assay using minimal media wheat cell wall fungal culture filtrates

Samples of culture filtrates from strain 22-8 and inter-specific hybrid DK did not reduce the wheat cell viability at any of the sample times. In contrast culture filtrates incubated for 12-30 days from strain 22-8 and culture filtrates incubated for 18-24 and 27-30 days from hybrid DK significantly ($P<0.05$) increased the fluorescence compared to the control samples. Culture filtrates from strain 22-8 generally increased fluorescence significantly more ($P<0.05$) than culture filtrates from hybrid DK (see Table 4.12).

Table 4.12: The reduction in wheat cell viability by minimal media wheat cell wall culture filtrates increasing in age from strains 22-8 and hybrid DK sampling every 3 days over a 30 day period. Transformed average pixel value presented.

Age of fungal culture filtrate (days)	Strain 22-8 Pixel Value	Inter-specific hybrid DK Pixel Value	Control Pixel Value
3	8.63	8.62	11.64
6	22.78	24.11	26.69
9	11.59	11.28	10.65
12	16.58 *	12.16	10.37
15	11.5 *	5.33	3.61
18	17.84 *	14.05 *	7.47
21	28.95 *	23.95 *	17.44
24	6.35 *	4	2.5
27	8.14 *	8.78 *	5.51
30	15.36 *	6.07 *	2.06

* Significant ($P<0.05$) increase in wheat cell viability compared to MYG media controls.

The pH of the fungal culture filtrates rose from pH 6.2 to approximately pH 7.5 for strain 22-8 and pH 6.9 for hybrid DK over the 30 days growth. The cultures appeared cloudy in appearance over time (Plate 4.4). Microscopic examination of the culture filtrates over the sampling times revealed microcyclic-conidiation to be occurring although some hyphae were also present. After 30 days incubation, strain 22-8 had produced approximately 6 times more spores (2.4×10^6 spores ml^{-1}) than hybrid DK (4×10^5 spores ml^{-1}).

4.3.13 Wheat root growth inhibition using minimal media cell wall fungal culture filtrates

No significant differences were found in wheat root growth when treated with fungal culture filtrates, control media or SDW (see Table 4.13).

Table 4.13: The effect of minimal media wheat cell wall fungal culture filtrates grown over a time course from strain 22-8 and hybrid DK on the average wheat root length.

Age of fungal culture filtrate (days)	Strain 22-8 Wheat root length increase expressed as % of cell wall control media	Inter-specific hybrid DK Wheat root length increase expressed as % of cell wall control media
3	96.5	83.6
6	102.8	105
9	114.24	91.3
12	113.22	106.08
15	96.35	103.54
18	95.74	80.78
21	101.76	86.78
24	89.88	80.39
27	83.19	78.17
30	81.5	70.33

* Significant ($P<0.05$) increase or decrease in wheat root length compared to MYG media controls.

Plate 4.1

Growth of strain and hybrid inoculum and inoculation of pathogenicity test plants.

A) Fungal inoculum growing on oat grain in mushroom spawning bags.

B) Inoculation of wheat plants with colonised oat grain inoculum (right hand pot) showing vermiculite (left hand pot) required to maintain a high humidity needed for infection.

A


OAT GRAIN INOCULATED WITH
PSEUDOCERCOSPORELLA
HERPOTRICHOIDES

B

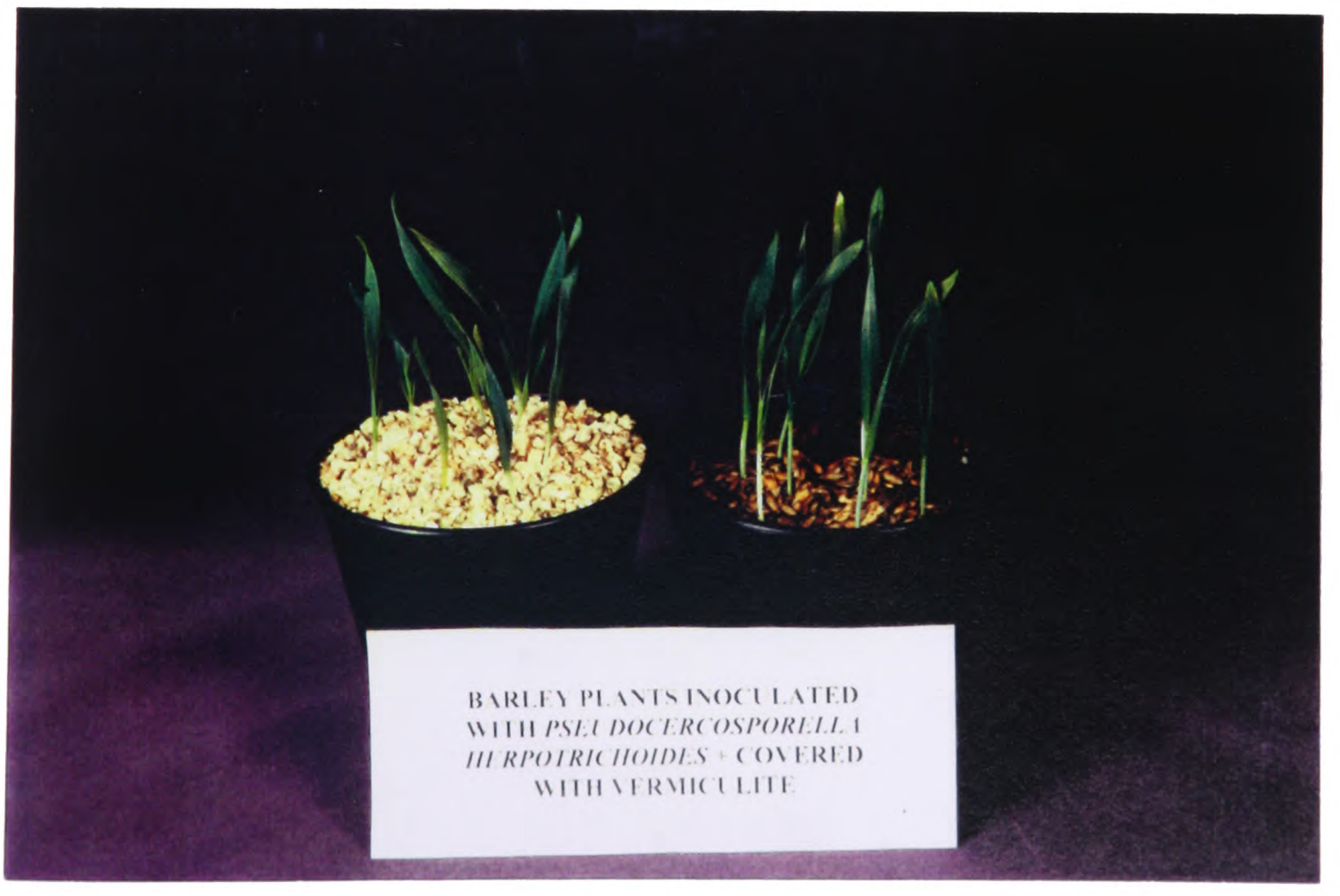

BARLEY PLANTS INOCULATED
WITH *PSEUDOCERCOSPORELLA*
HERPOTRICHOIDES + COVERED
WITH VERMICULITE

Plate 4.2

Pigmentation of colour mutants on maize meal agar.

A) Pigmentation of selected colour mutants on maize meal agar showing the underside of colonies shown. f = fulvous, o = olivaceous grey, b = buff, s = straw yellow, c = cream.

A

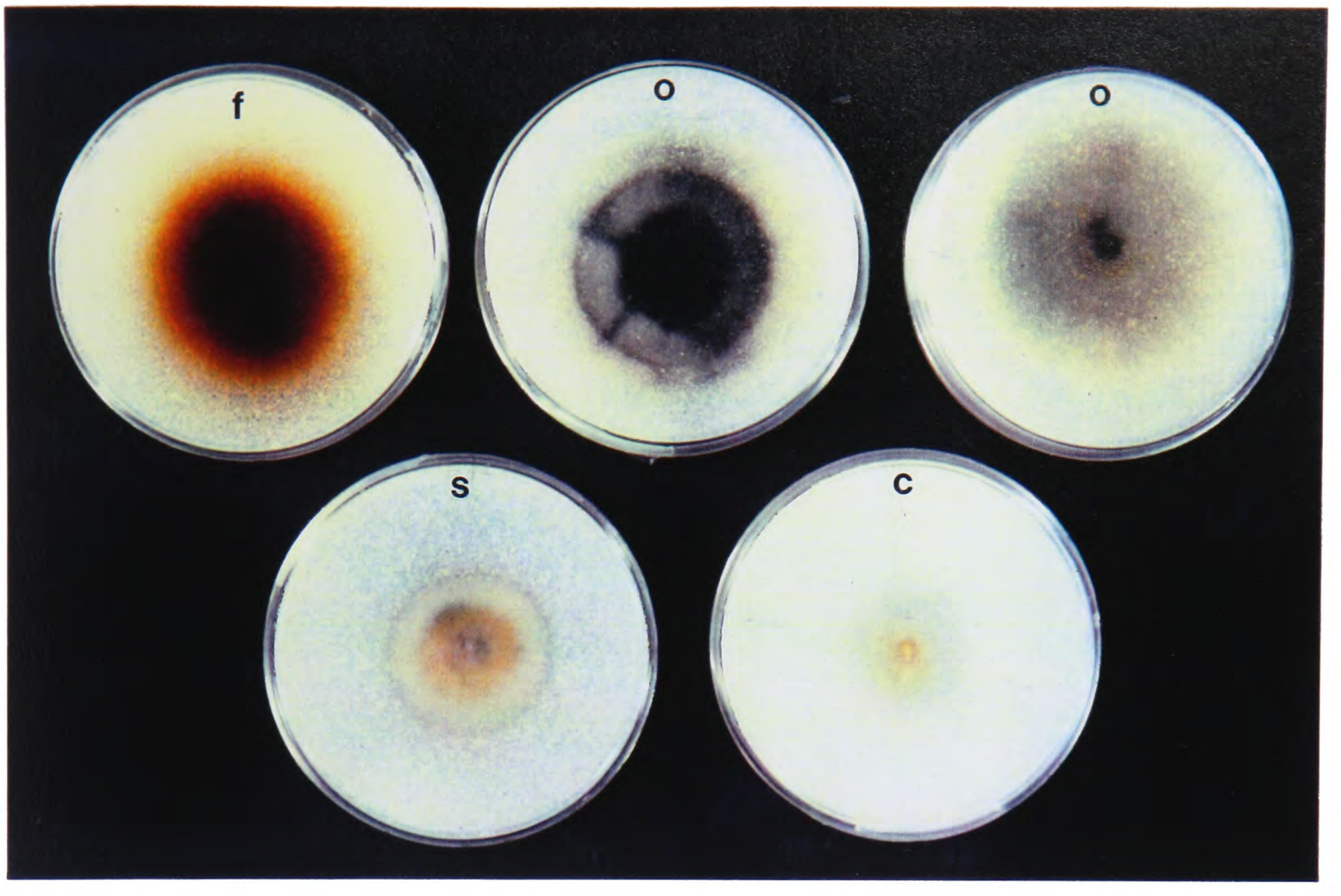


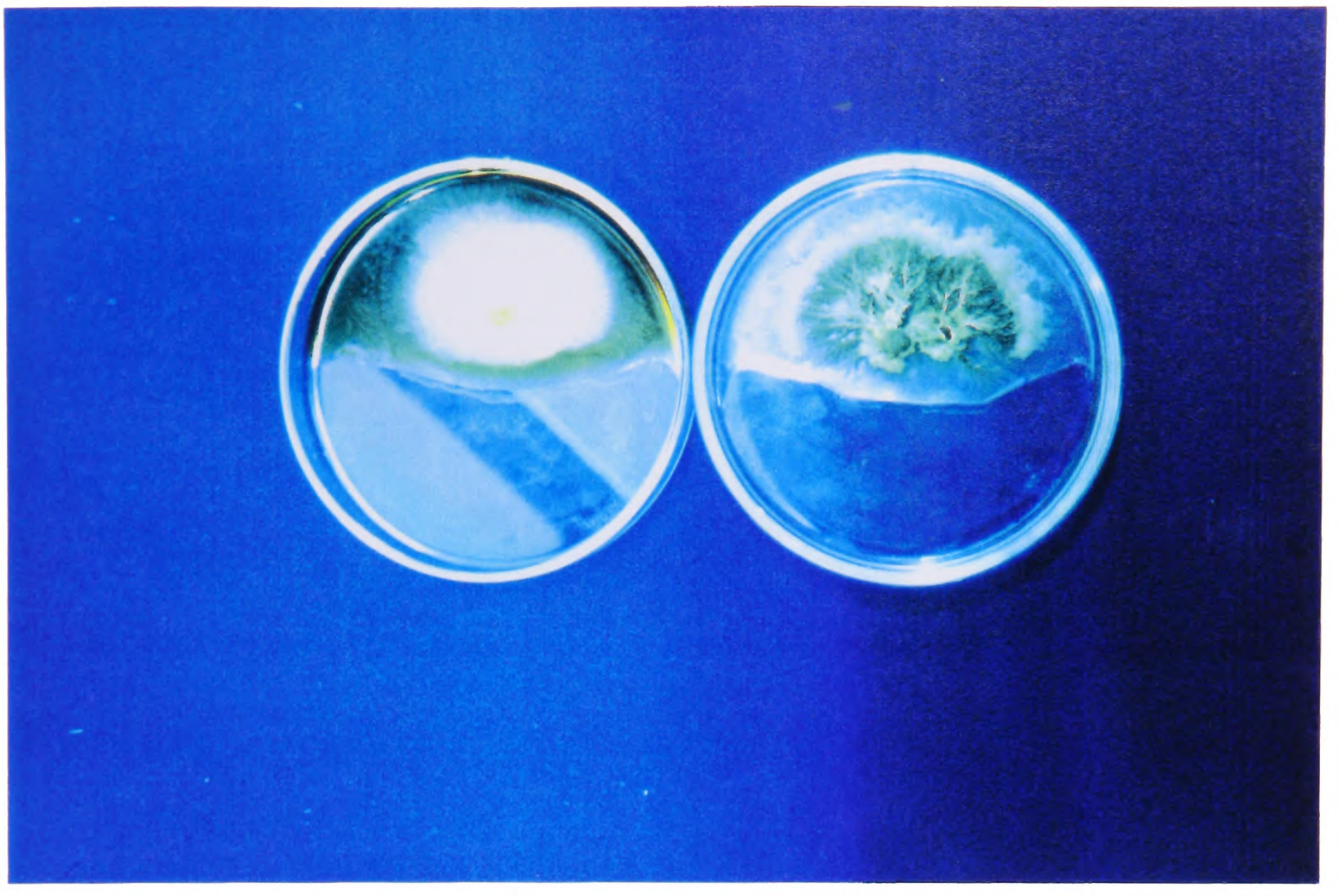
Plate 4.3

Infection plaque assay *in vitro*.

A) Sloped MYG agar in glass Petri-dishes inoculated with W x R hybrids of *P. herpotrichoides*.

B) Infection plaque produced by strain 22-119 on the bottom of the glass Petri-dish. Magnification x 400.

A



B

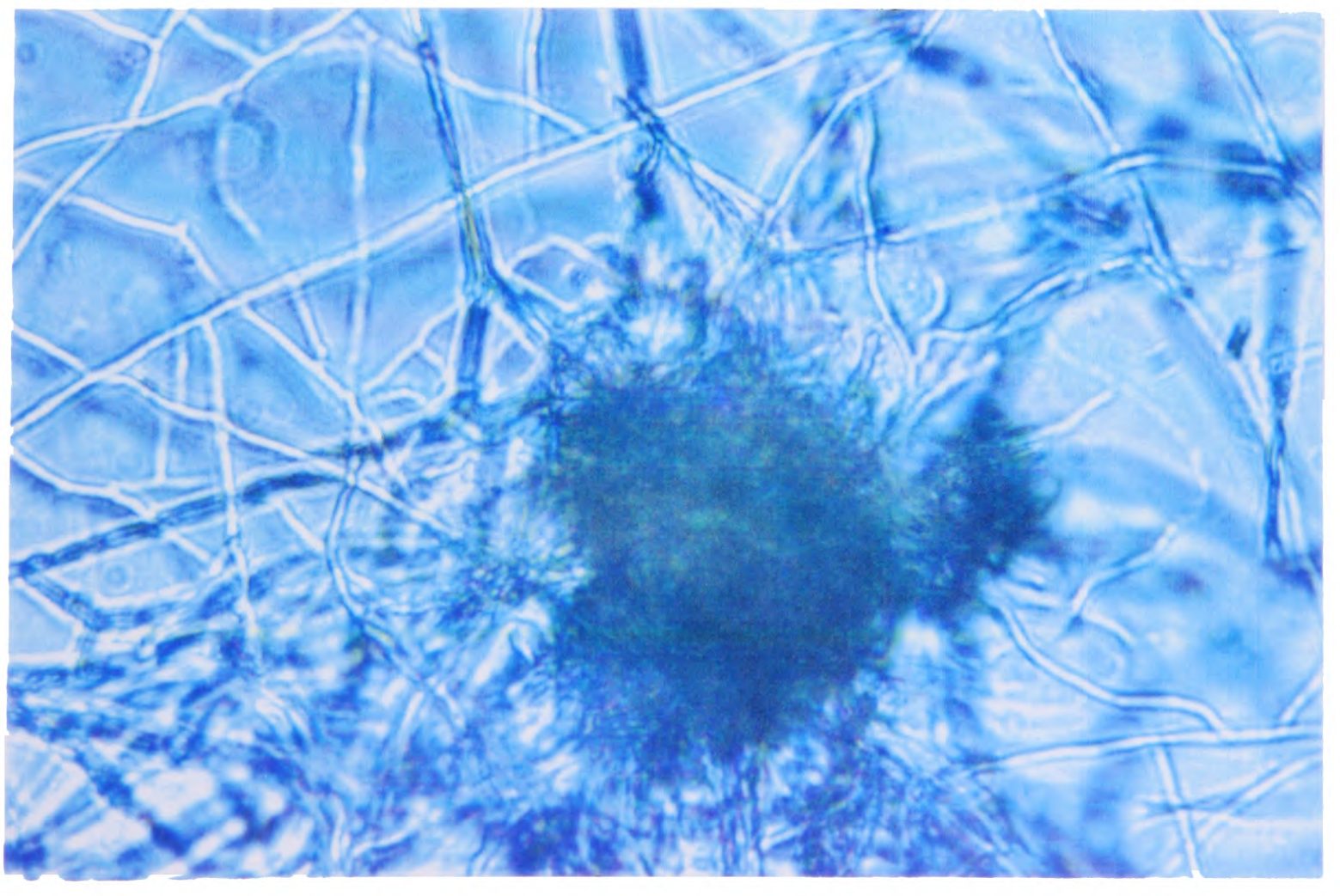


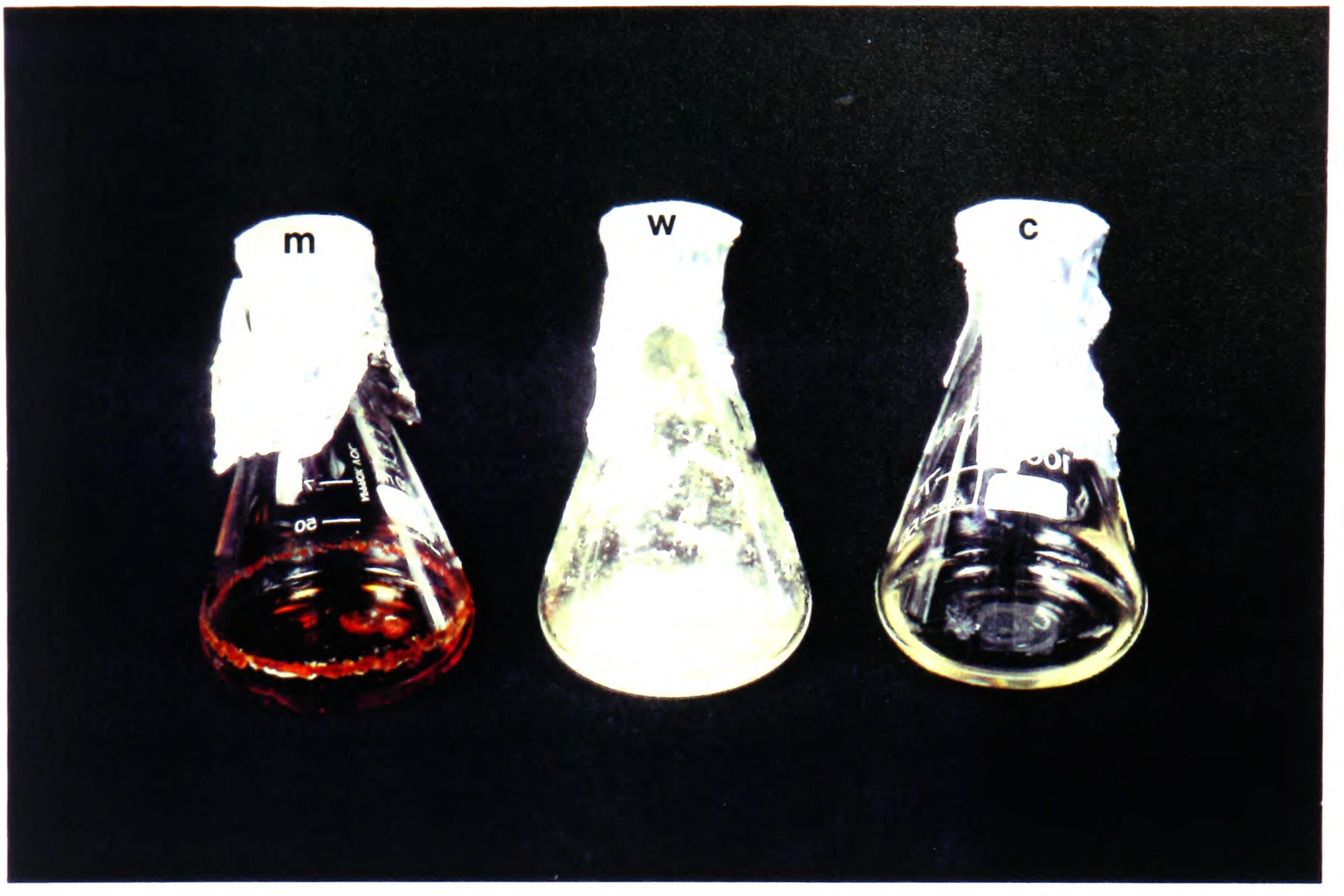
Plate 4.4

Detection of secondary metabolites using fungal culture filtrates and wheat cell suspension cultures.

A) Fungal cultures of strain 22-8 grown in MYG (m), minimal media containing a cell wall extract (w) and an uninoculated MYG control (c).

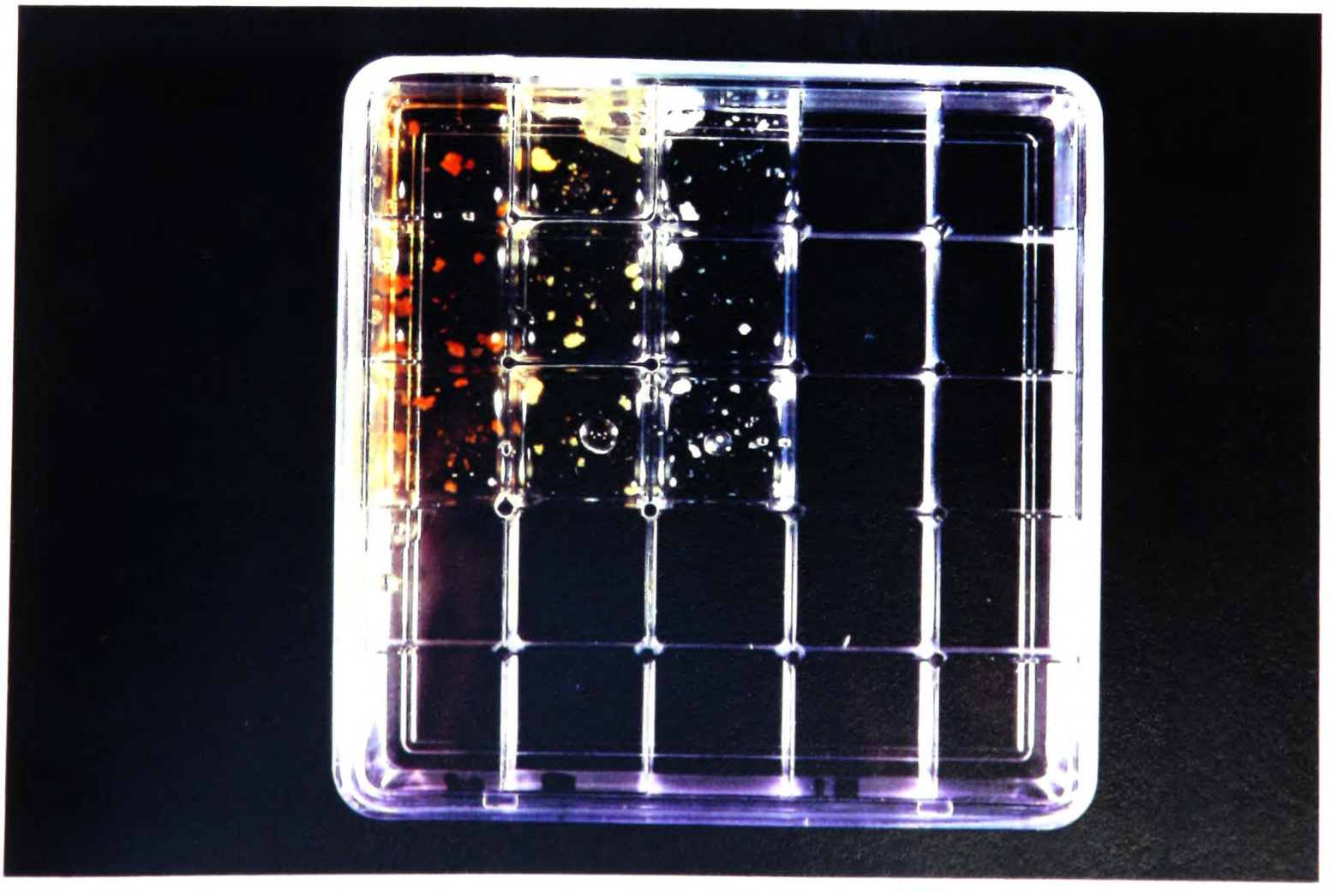
B) Wheat cells suspension cultures treated with culture filtrates from strain 22-8, hybrid DK and showing untreated cells. Viewed under natural light.

A



B

22-8 DK Control



4.4 Discussion

The colony morphologies of the W and R-type strains were similar to other characterised W and R-type strains, with the colonies having either even or feathery margins respectively (King and Griffin, 1985; Sanders *et al.,* 1986). The method of using colony morphology for identifying types of *P. herpotrichoides* has been shown to have some drawbacks. It has been observed that some cultures from R-type strains have an intermediate morphology, which produce sectors that can be fast growing with an even edge resembling the morphology of W-type strains (Hocart, 1987; Julian *et al.,* 1994). These sectors have been found to be of reduced pathogenicity and have altered fungicide sensitivity. Other intermediate morphologies cannot be readily classified as W or R-types without recourse to pathogenicity testing (Hollins *et al.,* 1985; Gallimore, Knights and Barnes, 1987). The W x R and inter-specific hybrid cultures displayed morphologies of either W or R-types but a few showed an intermediate morphology. The intermediate morphology may have been obtained through genetic recombination between W and R-types or it may be an R-type morphology which has sectored. Confirmation of the latter could occur by serial vegetative transfer, which has previously allowed for reversion back to the original morphology of some sectors (Julian *et al.,* 1994). All of the parasexual hybrids have been shown to be recombinants of their parents by techniques such as isozyme banding patterns (Hocart *et al.,* 1993a; McNaughton, 1996). Thus the morphological characteristics of the W x R hybrids may resemble either the W or R-morphology or a combination of both.

Typically W type strains are faster growing than the R-type ones (King and Griffin, 1985; Sanders *et al.,* 1986) but when characterising the W and R-type strains in the present work no difference in growth rates between the W and R-types was found. This may be due to continual serial vegetative transfer over many years altering growth characteristics. Some unusually fast-growing strains with feathery margins and unusually slow-growing isolates with smooth margins have been observed in the UK (Hollins *et al.,* 1985; Gallimore *et al.,* 1987) and it has been suggested that these

may be intermediate types or mixtures of colonies. Ascospores from *in vitro* and *in vivo* sexual crosses have been found to vary in colony morphology and may account for some of the morphological differences previously unexplained (Nicholson *et al.*, 1991a; Dyer pers. com., 1998). The W x R hybrids with W or R-morphologies however displayed different growth rates corresponding directly to W (fast/even) and R-morphologies (slow/feathery). The growth rates of all hybrids were less than the W and R-type strains which indicates either genetic change during parasexual recombination leading to reduce growth rates, or that the mutagenic treatments involving UV radiation used to obtain the original auxotrophic mutants used in the parasexual crosses may have given rise to other structural genome mutations in the parents. The growth rates of the hybrids showing the intermediate morphology were comparable to that of hybrids showing an R-morphology, supporting the idea that these colonies may be sectoring in culture. However currently nothing is known about the genetic basis of sectoring and it is just speculation. An alternative explanation for the slow growth rate of the intermediate morphology hybrids is that genomic inheritance from the R-type strain may be greater than the genomic inheritance from the W-type strain. The inter-specific hybrids appeared to have inherited the growth rate of *P. anguioides* and not the R-type parental strain. These results of colony morphology and growth rates of the strains and hybrids indicate that they are crude and often an inaccurate basis for differentiating pathotype characteristics.

Colour production on maize meal agar has been suggested to be a solution to the problems encountered when assessing colony morphology (Creighton and Bateman, 1988; Creighton, 1989). In the present work the underside of maize meal agar grown colonies of R-type strains were fulvous and W-type strains olivaceous in colour agreeing with convention (Creighton and Bateman, 1988; Creighton, 1989). The W x R hybrids showing either W and R-morphologies displayed the fulvous colour of the R-type parent, while the hybrids with the intermediate morphology also appeared predominantly fulvous in pigmentation. This suggests that R-type pigmentation on

maize meal agar was inherited preferentially to the W-type pigmentation on the same substrate. As some hybrids had a colour classification different to that expected from their colony morphology it indicates that the genes for colony morphology are separate from those for pigmentation on maize meal agar.

The inability of 13 of the hybrids to produce conidia may be attributed to them losing or having altered the genes involved in the stimulus/production of conidia during parasexual recombination or initial mutagenic treatments as mentioned previously. As no differences in spore dimensions between the W and R-types (Fitt *et al.*, 1987) and from observations of W x R hybrid conidia (McNaughton, 1996) it was shown that the hybrid conidia did not vary in dimensions. The longer spores from the inter-specific hybrids confirm the observations of Hocart and McNaughton, (1994) who found that hybrid spores were longer than that of *P. herpotrichoides* but shorter than that of *P. anguioides*.

Molecular characterisation using *Hae III* digests on ITS products showed that the W and R-types strains have different banding patterns. Restriction digests of other non-specific PCR products from the ITS region of the ribosomal genes have also classified strains of *P. herpotrichoides* into 2 groups corresponding to W and R-types (Gac *et al.*, 1996). The enzyme digests of the ITS product from the selected W x R and inter-specific hybrids tested showed banding patterns only of an R-type, indicating that the ITS region of the nuclear rDNA in these hybrids is from the R-type parents. This also supports segregation in favour of the R-type parental genome in these hybrids. Further studies could examine the ITS regions of the hybrids having an intermediate morphology. The ITS primers used here amplified the non-coding regions between the conserved regions of the 18S, 5.8S and 28S ribosomal ribonucleic acid (rRNA) genes. The ITS region evolves at a fast rate and these high levels of divergence of the ITS region have been utilised for the development of PCR assays that are able to differentiate and analyse phylogenetic relationships of many

different fungal species, within a genus or among populations (White *et al.,* 1990; Li, Rouse and German, 1994; Arora, Hirsch and Kerry, 1996).

The specific primers TaO5F/R and Ty16F/R, originally developed from RAPD amplification products common to either W or R-type strains (Nicholson *et al.*, 1997), were also used to confirm the identity of the W and R-type strains in this experiment. The W x R -specific hybrids which were tested with both primer pairs showed both W and R amplification products. These specific primer sequences amplify unknown W and R genomic regions but show that the W x R hybrids have recombinant genomes. The inter-specific hybrid (R-type x *P. anguioides*) only amplified the R-type fragment indicating the presence of the R-type parent but as the primers were designed to be specific for W and R-type strains no amplification product from *P. anguioides* would be expected. Priestly *et al,* (1992) found that isozyme banding patterns of *P. anguioides* were closer to that of the R-type strains than the W-type strains and segregation of parental markers during parasexual recombination showed a bias to the R-type genetic marker being present in the hybrids (Hocart and McNaughton, 1994). This supports the mainly R-type morphological and molecular characteristics of the inter-specific hybrids although only a small number of markers are available for characterisation in these crosses. Specific primers have also been developed from sequences of the ITS region amplifying differentially DNA from W and R-types (Poupard *et al.,* 1993). These primers could be used along with RFLP of total DNA, rDNA, mitochondrial (mt)DNA and RAPDs on the hybrids and would aid understanding of the gene segregation from the parental strains. The use of nuclear (n)DNA re-association kinetics has found a low DNA relatedness between groups of W and R-types and between both groups and strains of *P. anguioides* (Takeuchi and Kuninaga, 1994). The nDNA relatedness would also be useful tool in characterising the W x R and inter-specific hybrids to their parental genomes.

These molecular techniques support the cultural characterisation studies but are not as time consuming or unreliable. They also show the inheritance of specific genomic areas and assist in the characterisation of the W x R- and inter-specific hybrids.

The pathogenicity on wheat, barley and rye of the parental W and R-type strains and hybrids was determined by the depth of penetration through the stem of the disease symptoms. The W and R-type strains showed varying pathogenicity to wheat, barley and rye with the W types displaying higher mean disease infection scores on wheat and rye than the R types. This is in contrast to other findings where both the W and R -types have been found to be equally pathogenic to wheat and the R-type significantly more pathogenic to rye (Lange-De-la-Camp, 1966; Scott *et al.,* 1975; Nirenberg, 1981). In addition, other studies have found that R-types can be more pathogenic to wheat than the W-type (Hollins *et al.,* 1985). In this work the decrease in pathogenicity of the R-type strains on both wheat and rye may be attributed to continuous *in vitro* transfer of the strains over several years although the W-type strains had also been in culture as long. Studies have shown that different environmental conditions can affect pathogenicity. Wheat seedling pathogenicity tests have found that at 10°C, W and R-types are equally pathogenic (Brown *et al.,* 1984), R-types are more pathogenic than W types at 7°C (Hollins *et al.,* 1985) and W-types are more pathogenic than R-types at 18-22°C (Higgins and Fitt, 1985a; Sanders *et al.,* 1986). The day temperature in the glasshouse during this experiment was 17°C which would suggest increased pathogenicity of the W-type compared to the R-type on wheat seedlings. The minimum night temperature was 2°C but this may not be as important as the day temperature since increasing the temperature has been found to increase the rate of penetration through the stem base (Ponchet, 1959). It has been found however that a cool night temperature is important for symptom induction in both wheat and rye (Hocart pers. com., 1998). The pathogenicity data in the present study also suggests a similar relationship might exist between temperature and infectivity on rye although no experiments have been published relating to this.

Variation of the disease symptoms was seen to occur within a single pot but this may have been due to the inoculation technique. The amount of inoculum that was placed around the stem base of each plant was not standardised because it was a clump of infected oat grain, which may or may not have given an even distribution of the strain growing throughout the whole. Auxotrophic mutants were amongst the hybrids assessed and the ability of these to grow on oat grain in the mushroom spawning bags may have been impaired due to the lack of certain amino acids. Previous findings on the pathogenicity of the inter-specific hybrids was thought not to be impaired by the presence of certain auxotrophic markers (Hocart and McNaughton, 1994). Indeed in this assessment the inter-specific hybrids DK, DQ and DM, all cysteine auxotrophic strains, showed increased pathogenicity to wheat, barley and rye compared with the parental R-type (22-12). As symptoms were seen for many of the hybrids and W and R type strains it indicates that conditions were favourable for infection and the lack of pathogenicity seen in other progeny can be attributed to other factors.

Analysis of the pathogenicity of the W x R and inter-specific hybrids showed that they varied in their pathogenic ability to infect wheat, barley and rye. In the studies by McNaughton, (1996) no correlation was found between the ability of the hybrids to infect wheat, barley or rye and it was suggested that the ability to cause the disease on these hosts is separately controlled. In this study many hybrids were of low pathogenicity or non -pathogenic to all 3 hosts. Low mean disease infection scores recorded as 1 may not have been disease but natural senescence of the coleoptile expressing itself as slight brown necrotic areas; this was typically seen in the visual assessments made on barley. Where moderate or high pathogenicity was observed in the hybrids novel pathotypes were seen (i.e. high pathogenicity on rye but non-pathogenic on wheat) although this only occurred for a couple of the hybrids. Infection by a strain or hybrid classed as having low pathogenicity on any of the hosts may result in it being non-pathogenic, asymptomatic or of low pathogenicity depending on the other hosts (wheat, barley or rye) and infection conditions.

The pathogenicity of the strains and hybrids to barley could not be correlated to their pathogenicity to wheat or rye. Generally hybrids showing low or non-pathogenicity to barley were of a low pathogenicity or non-pathogenic to wheat and rye. Therefore from these studies it is suggested that pathogenicity is separately controlled on the 3 hosts. However because very few hybrids showed a novel pathotype this is not conclusive.

Infection plaques identified on the first three leaf sheaths were similar in morphology to those described by Daniels *et al,* (1991). These plaques are swollen hyphal cells that are either loosely associated or discrete and circular closely associated. A strain or hybrid showing high pathogenicity to a host had more infection plaques than a strain of low pathogenicity on that host. The distribution of the infection plaques on the three leaf sheaths examined showed that more infection plaques were present on the first leaf sheath compared to the third leaf sheath. These results indicate that the ability of the strains to produce the infection plaques relates to successful penetration of the leaf sheaths. Unsuccessful penetration may be attributed to the lack of ability of the strains to form functional infection plaques or the formation of these infection plaques is at a reduced rate that will allow the host plant to activate resistance mechanisms to contain the fungus. Alternatively natural senescence of the coleoptile and first leaf sheath may occur before the fungus has infected the next tissue layer thus preventing further penetration of these 'slow plaque forming' strains/hybrids through the stem.

Microscopic examination of plant tissues classified several hybrids as being asymptomatic. These strains showed no disease symptoms on the plants. Hybrids P262, D3/41 and Z449a were asymptomatic on wheat, hybrids 128I and AI382 were asymptomatic on barley and hybrid O251 was asymptomatic on rye. There were fewer infection plaques produced by the asymptomatic strains compared to the pathogenic strains and this indicates that pathogenicity factor(s) such as cell wall degrading enzymes may be missing. These factors may be directly causing the

disease symptoms or reduce the ability of the strain to infect and colonise the host. Pathogenicity has been shown to be heritable (Hocart and McNaughton, 1994) but in the current study a large proportion of the hybrids were found to be non-pathogenic or of low pathogenicity to the three hosts. This may be attributed to mutagenic treatments and the process of parasexual recombination as mentioned earlier which may have significantly disrupted the genome of these hybrids. The hybrids classed as being asymptomatic on one host species were of low or non-pathogenicity to the other two hosts. This indicates that they are of low pathogenicity generally and altering the environmental conditions or even repeating the experiment may change on which host infection plaques are seen and the presence of any disease symptoms. Common requirements for pathogenicity to these three hosts may be absent in these hybrids of generally low pathogenicity.

When an assessment is made of pathogenicity of strains and hybrids it is essential to carry out a visual assessment of disease symptoms, and a microscopic examination of the stem tissue for infection plaques to identify both pathogenic and asymptomatic strains.

Classification of the strains by pathogenicity testing would have grouped the strains of high pathogenicity to wheat and rye as R-types and those strains of low pathogenicity to wheat and rye probably as W-types. This however is an incorrect classification of them, disagreeing with the cultural and molecular techniques previously used. It also demonstrates how labour intensive pathogenicity testing is and how it can be influenced by many variables such as the environment which can give misleading results. The pathogenicity classification of the hybrids could not be correlated with their cultural/molecular classification, this would be expected as they are genetic recombinants. As an example, strain 138I showed pathogenicity of a classical W-type, morphologically was a W-type, but its pigmentation on maize meal was fulvous of an R-type.

The ability of the strains and hybrids to produce infection plaques was examined *in vitro* using glass-Petri-dishes containing a slanted agar medium. Infection plaques were produced after the edge of the agar slant which indicates that a lack of nutrients and/or a change in texture from an agar based medium to glass induced the formation of the infection plaques. A lack of nutrients, mechanical cessation of growth and the presence of a solid surface has previously been shown to induce infection plaques from *P. herpotrichoides* when grown in agar and liquid culture (Deacon, 1973). In his studies Deacon showed plaque development only occurred if an adequate energy source was available. Similar findings occurred here in preliminary studies where full strength MYG produced more infection plaques than half strength MYG. The infection plaques appeared as swollen cells from sub-terminal branching of hyphae which are similar in appearance to those characterised by Deacon (1973).

The W-type strains produced moderate numbers of plaques and the R-type strains produced a high number of infection plaques *in vitro.* All the infection plaques produced by both W and R-types varied in size. Infection plaque production *in vitro* could be correlated generally to pathogenicity. The R-type strain 22-8 produced many infection plaques *in vitro* and was of high pathogenicity to wheat and barley and of lower pathogenicity to rye, the W-type strain 22-20 produced fewer infection plaques *in vitro* and was of a high pathogenicity to wheat but lower pathogenicity to barley and rye. Strains 22-1 (W-type) and 22-119 (R-type) produced high numbers of infection plaques *in vitro* and were highly pathogenic to all 3 cereal hosts. It was generally found that if a W x R or inter-specific hybrid showed high pathogenicity to wheat, barley or rye such as inter-specific hybrids DK and DM they produced a high or moderate number of infection plaques *in vitro.* Infection plaque size varied between the hybrids but did not correlate with pathogenicity. Those hybrids classed as being of low pathogenicity, asymptomatic or non pathogenic on the cereal host species produced few or no infection plaques *in vitro.* This corresponds with infection plaque numbers seen *in vivo* on infected host tissue and indicates that

infection plaques are probably an important factor in determining the pathogenicity of the strains or hybrid.

Infection plaques *in vivo* are initiated from adventitious runner hyphae growing from mycelial plates (Daniels *et al.,* 1991), the exact stimulus for infection plaque production is however unknown although a lack of nutrients would be plausible. Morphologically similar, discrete infection structures have also been found in *Rhizoctonia solani* Kuhn. Here the principal stimulus is the topography of the hypocotyl in which host signals such as plant exudates are required for the production of mucilage necessary to promote close adhesion to the plant surface (Armentrout *et al.,* 1987). The infection plaque cells produced by *P. herpotrichoides* in contact with the host produce an infection vesicle at the tip. Erosion of the host cuticle and cell wall softening through the liberation of cell wall degrading enzymes leads to penetration of the host. Daniels *et al,* (1991) found that weakly pathogenic strains of *P. herpotrichoides* on wheat seedlings had penetration inhibited by deposition of osmophillic material in the host cell walls beneath the plaques whereas in highly pathogenic strains the host cell wall was unmodified. The cells of the infection plaques effectively behave as compound appressoria similar to those which are found in *Botrytis cinerea* Pers.: Fr (Backhouse and Willetts, 1987) promoting rapid host colonisation. This supports the importance of infection plaque production and viability in pathogenicity.

The infection plaques produced *in vitro* appeared to have less structure (swollen cells more randomly arranged) than those seen on infected host tissue. This could reflect the different stimulus *in vitro* and different availability of nutrients and conditions of growth. Thus the ability to produce infection plaques *in vitro* may reflect the general ability of a strain/hybrid to infect a cereal host but not the direct relationship between host species and strain pathogenicity.

Disease symptoms caused by infection by *P. herpotrichoides* are necrotic lesions found on the stem base and, examination under the microscope of these lesions shows the presence of fungal structures such as infection plaques, mycelial plates and runner hyphae. The lesion consists of necrotic host cells, many of which are not in contact with any fungal structure but several cells away from them (Hocart pers comm, 1986). This perhaps suggests that there may be diffusion of toxic materials from the fungus which is killing the host cells although they are not in contact with the fungus. Alternatively it could be a host cell response to fungal elicitors such as glucans or chitosan causing the hypersensitive reaction leading to cell death, or related to this, signals produced from the host cells in contact with or infected by the fungus causing cell death in the surrounding cells. There is however no published evidence of the hypersensitive response in *P. herpotrichoides* interaction with host cells.

The search for secondary metabolites which may lead to host cell death was performed by examining the viability of wheat cells after the addition of fungal culture filtrates. Cell suspension cultures were used as the cells have no cuticle and therefore the effect of any toxin/enzyme/elicitor would be more easily detected. Protoplasts could also have been used although this leads to more experimental complexity and is further away from *in vivo* host plant/pathogen interactions. Many variables that operate in glasshouses and the field can be eliminated using cultured cells. The cells are aseptic and unchallenged by any microflora and are undamaged by any biotic agents. Culture filtrates may contain other materials that are toxic to cultured plant cells although no evidence of them was found in the different fungal growth media used here.

Reduced wheat cell viability was seen when treated with culture filtrates from fungal growth in MYG. This indicates that these filtrates of MYG contained something which reduced wheat cell viability. This material could be an enzyme, toxin or fungal elicitor secreted into the media which appeared only to be present once the fungal

cultures had reached a stationary phase in growth, as a reduction in wheat cell viability was not seen before 18 days of growth. A corresponding assay using wheat roots found no inhibitory effect of the MYG culture filtrates. This suggests that either wheat roots were not susceptible to this substance or that what is apparently reducing wheat cell viability is not actually toxic. Approximately 12 days after inoculation fungal pigments started to appear in the media and a build up of these pigments was seen to coincide with reductions in wheat cell viability measured using fluorescein diacetate. A visual and microscopic examination of the culture filtrate treated wheat cells found that the fungal pigments appeared to be present in the plant vacuoles and it is probably this that was the cause of reduction in fluorescence and not wheat cell death (although cell death caused by the pigments cannot be completely ruled out). Further evidence confirming this is that the reduction in wheat cell viability can be correlated with the amount of pigments present in the culture filtrate. Strain 22-20 produced few pigments and its culture filtrates did not reduce wheat cell viability. All other strains and hybrids tested produced pigments and reduced wheat cell viability, the extent of which appeared to be correlated with the intensity of pigment production into the medium. To verify that it is the pigment that causes a reduction in fluorescence, the pigment could be removed using Thin Layer Chromatography and then added directly to the wheat cells. In future work a hydrophobic column could be used to separate out components of the fungal culture filtrate to remove the pigments. The filtrate minus the pigments could then examined for its effect on wheat cells. Strains 22-20 and 22-8 and hybrids P262, O251, C41 and DK all varied in their ability to cause disease symptoms with strains 22-20 and 22-8 and hybrid DK being of high pathogenicity to wheat cells (thus strong disease symptoms) and hybrids O251, P262 and C41 were of low pathogenicity, asymptomatic and non-pathogenic respectively on wheat. All the strains and hybrids except for strain 22-20 reduced wheat cell viability and produced pigments in the medium. This indicates that it is the pigment causing the effect of reduced wheat cell viability and probably not a substance produced only by the pathogenic strains/hybrids.

Specific host toxins have been found to be generally produced by the host species under relatively normal growth conditions for culturing the pathogen *in vitro*. Cercosporin, the toxin from *Cercospora oryzae* Miyake is produced in a medium of Czapek-Dox or potato sucrose agar and can be extracted in diethyl ether (Batchvarova, Reddy and Bennet, 1992). In some instances an activator for toxin production is required for example in *H. sacchari* either a sugar-cane leaf extract or serinol is required to induce HS toxin production in culture (Larkin and Scowcroft, 1981). Neither the full strength MYG media used for normal culture of *P. herpotrichoides* or a cell wall based medium induced the production of clearly toxic substances. The absence of a signal/elicitor cannot be ruled out, or that the growth conditions used in this experiment may have made any toxic substance present unstable. Alternatively the fungal substance could have interacted with other substances present in the media. Fluorescein diacetate has been used successfully to detect cell viability of calli treated with the toxin Cercosporin (Batchvarova *et al.*, 1992), and cell suspensions from resistant cultivars of chickpea (*Cicer arietinum*) turned brown when treated with a protein elicitor from culture filtrates of *Ascochyta rabiei* (Pass.) Labrousse (Vogelsang *et al.*, 1994). A retardation of root growth has been found with oat roots treated with HV toxin from *C. victoriae* (Luke and Wheeler, 1955) and similar effects were seen in sorghum roots treated with PC toxin from *P. circinata* (Pringle and Scheffer, 1963). The lack of root inhibition and questionable reduction in wheat cell viability suggests that *P. herpotrichoides* does not produce any toxins under the growth conditions tested.

The identification of toxic substances can be used in the selection of resistant plants. Culture filtrates from the fungal pathogen *F. oxysporum* f. sp. *medicaginis* (Weimer) Snyder & Hansen have been used to select resistant plants of alfalfa (Hartman, McCoy and Knous, 1984), and filtrates from *F. oxysporum* Schlecht have inhibited wheat callus tissue growth (Shayakhmetov and Asfandiyarova, 1991). Girko *et al,* (1993) examined culture filtrates from *P. herpotrichoides* grown in a medium of potato, sugars and vitamins and used them to select resistant calluses of wheat. There

was however no mention in this paper of the detection/identification of any toxic substances in the culture filtrates they used. Cell suspensions have also been used for agrochemical research for screening plant growth retardants and herbicides for activity by monitoring cell division (Grossmann, Berghaus and Retzlaff, 1992) and to select cells tolerant to environmental stress such as freezing, heat and salt (Ishikawa, Robertson and Gusta, 1995).

An increase in fluorescence was seen in the wheat cells treated with culture filtrates from strain 22-8 and hybrid DK growing on minimal media containing a wheat cell wall extract as the sole carbon source. A minimal medium containing cell wall extract was used to try to simulate closer the conditions found on the host plant which might induce secondary metabolite production. The increase in fluorescence seen may have been due to an uneven distribution of wheat cells in the wells when the image analysis was performed but this is unlikely to occur repeatedly. A more likely reason for the increase in fluorescence could be that the fungal enzyme secreted into the media may be directly cleaving the fluorescein diacetate and thereby enhancing the fluorescence. Alternatively, it is possible that wall degrading enzymes such as pectinases or glucanases might have been produced by the fungus in culture. These enzymes may be digesting the middle lamellae of the wheat cells thus breaking the cell clumps apart and increasing the surface area of activity to absorb fluorescein diacetate. The greater increase in fluorescence produced by strain 22-8 compared to hybrid DK may be due to the better growth of the former strain in the medium, which produced a greater number of spores thus producing more enzymes capable of breaking apart the wheat cell clumps. Pectolytic enzymes such as polygalacturonase, pectin lyase and pectin methyl esterase at very low levels, and arabinase, xylanase, laminarinase at higher levels, along with celluloytic enzymes have been shown to be produced when *P. herpotrichoides* is grown in liquid culture. This contained either citrus pectin or wheat cell walls from straws, and production occurred after 1-2 days growth (Hanssler *et al.,* 1971; Cooper *et al.,* 1988). These enzymes have also been detected during the infection of wheat with strains of *P. herpotrichoides,* the enzyme

activity being found to increase with increasing temperature with the highest activity at 20°C (Mbwaga *et al.,* 1997). Cell wall degrading enzymes are suggested to be produced when penetration occurs of host cell plants via infection plaques (Daniels *et al.,* 1991). Addition of the culture filtrates to potato discs and monitoring the ease of which the discs fall apart would be a simple method to detect pectinases present in this system. Xylanases, arabinase and laminarinase could also be assayed using the substrates xylan, araban and galactan by the determination of reducing groups from these substrates (Cooper *et al.,* 1988). The pH optimum of polygalacturonase is pH 4.0-5.0 (Hanssler *et al.,* 1971). However the pH of the minimal medium containing cell walls was 6.2-7.5, hence buffering this medium may allow better detection of the enzymes. As wheat cell walls and glucose were used as the carbon source, varying the carbon source by growing on cellulose, xylan, pectate and pectin has been found to alter cell wall degrading enzymes produced by *P. herpotrichoides* (Cooper *et al.,* 1988). Their studies also suggested both induced synthesis and constitutive synthesis of the different enzymes. The monomer of the substrate for the enzyme has also been found to be required for enzyme induction (Cooper and Wood, 1975). A lack of monomer unit or excess may lead to non-induction or catabolic repression respectively of the desired enzyme, both of which could have occurred here particularly with the fungal cultures grown in MYG. The virulence of isolates of *V. albo-atrum* has been related to their *in vitro* secretion of cell-wall degrading enzymes (Carder, Hignett and Swinburne, 1987). Specific studies directly targeted at cell-wall degrading enzymes would determine their role in the pathogenicity of the strains and hybrids classed as being pathogenic, asymptomatic or non-pathogenic.

The current work demonstrates the different methods which can be used to characterise and identify both the W and R-types and parasexual recombinant hybrids. The methods used additionally confirm that the parasexual progeny are recombinant hybrids. The pathogenicity of the hybrids on wheat, barley and rye suggests that pathogenicity is separately controlled for these hosts and, the numbers of infection plaques produced on the first three leaf sheaths of the host can be related

to the pathogenicity of the strain or hybrid. The ability to produce infection plaques *in vitro* enabled them to be related to strain/hybrid pathogenicity *in vivo*. Final studies using hybrids classed as being of high or low pathogenicity, or asymptomatic to wheat were unsuccessful in detecting any secondary metabolites which may be involved in pathogenicity.

CHAPTER 5

A COMPARATIVE STUDY OF THE RELATIONSHIP BETWEEN FUNGAL BIOMASS, FUNGAL PROGRESS AND DISEASE SYMPTOM DEVELOPMENT

5.1 Introduction

Traditional disease assessments of *P. herpotrichoides* on wheat, barley and rye differ from those of many of the foliar and root diseases because disease severity has been assessed not as the area affected but by the depth of penetration of the lesions at the stem base. Thus for leaf sheath lesions, a severity score based on the number of leaf sheaths infected or penetrated has been used (Scott, 1971). Microscopic studies have revealed that on wheat seedlings the mode of infection to the first leaf sheath differs between the W and R-types (Daniels *et al.,* 1991). In addition, field experiments have indicated that the epidemiology of the two types might also differ with the later appearance of symptoms on wheat stem bases by the R-type compared to the W-type (Cavelier, Rousseau and Le Page, 1987; Goulds and Fitt, 1990; Bateman, 1993; King and Griffin, 1985; Coskun *et al.,* 1987). The development of quantitative PCR has permitted the proportion of a pathogen in a single plant to be estimated. This is based upon the relative degree of amplification of the target DNA versus a competitor DNA fragment within each sample. Specific PCR primers developed to identify W and R-types used in conjunction with respective competitor DNA fragments have allowed comparisons to be made of the amounts of fungal DNA present in wheat and rye infected tissue in glasshouse and field experiments (Nicholson *et al.,* 1997). The aim of the work described in this chapter was to relate fungal biomass as estimated by DNA quantification to fungal progress (microscopic assessment for infection plaques, hyphae etc.) and symptom development in strains and hybrids with contrasting pathogenic abilities.

5.2 Material and Methods

5.2.1 Strains and hybrids

The parental strains W-type 22-20 and R-type 22-8, W x R hybrids O251, P262, C41 and the inter-specific hybrid DK (see Plate 5.1) were chosen because of their contrasting pathogenic, asymptomatic or non-pathogenic abilities on wheat and rye as determined previously (Chapter 4).

5.2.2 Production of conidia

To check the conidial viability and allow standardisation of inoculation procedures a 1ml aliquot of a 2 x 10^5 ml^{-1} spore suspension in SDW (supplemented with 50 µg ml^{-1} citrulline, due to the presence of auxotrophic arginine hybrid O251 unable to make citrulline) was placed on to MYG plates and incubated at 19°C in the dark for 24 hours. Five hundred conidia were assessed using a binocular microscope and determined viable if germ tubes were present from the ends of the conidium. The assessment had 4 replicates.

5.2.3 Growth of plants

Wheat cv. Beaver and rye cv. Halo seeds were sown in 6 cm^2 plastic pots filled to within 2 cm of the rim with peat based compost, 5 seeds per pot. Plants were maintained in plastic trays placed in Fitotron growth cabinets (16 hour photo-period, day temperature 12 ± 2 °C, night temperature 6 ± 2 °C, 80% relative humidity) and watered daily.

5.2.4 Inoculation of plants

Fourteen day old seedlings were used for inoculation. Three cm long pieces of polyvinyl tubing (internal diameter 4mm) were placed over the wheat seedlings and pushed 1cm down into the soil, enclosing the seedling base. A 1.5ml aliquot of a 2 x 10^5 ml^{-1} spore suspension in SDW (supplemented with 50 µg ml^{-1} citrulline) was pipetted into each tube and allowed to drain into the soil. Watering of the seedlings prior to inoculation improved the spore distribution over the coleoptile. After

inoculation the pots were filled with vermiculite to maintain a high humidity around the stem base needed for infection. After 72 hours the pieces of polyvinyl tubes were removed to prevent constriction of the stem base later on. Control plants were inoculated with SDW containing 50μg ml^{-1} citrulline (see Plate 5.1).

5.2.5 Pathogenicity assessment

The experiment was carried out over 16 weeks (8 weeks wheat and 8 weeks rye) with 3 replicate pots per strains per sampling time period, sampling occurring every week. Wheat pathogenicity assessment took place in the first half of the period and rye pathogenicity assessment took placed in the second half. The pots were randomly allocated in a block design in two cabinets. Pots were watered by filling the propagation trays in which they stood with water as required to avoid overhead watering thus preventing cross contamination between pots.

5.2.6 Disease symptom assessment

A visual assessment of pathogenicity was made using the infection scoring system described by Scott (1971) on 3 of the pots (total 15 seedlings) which is summarised in Section 4.2.12. The data was analysed using the Kruskal-Wallis test for non-parametric data as described in Section 4.2.14.

5.2.7 Microscopic assessment

A microscopic assessment was made, for the presence of infection plaques, hyphae and secondary sporulation, with the fluorochrome ANS staining of all surface structures. Five of the stem bases from one cabinet were examined by uprooting the seedlings and removing the coleoptile and subsequent leaf sheaths. These tissues were placed on glass microscope slides and stained as described in Section 4.2.13. The total number of infection plaques on the coleoptile and each leaf sheath were thus determined.

5.2.8 Sample preparation for DNA quantification

To determine the quantity of fungal DNA in the stem bases 2 replicate pots (5 plants per pot) were used. The roots were removed as close as possible to the crown and a 4cm section of stem base was cut from above the crown, the upper part of the plant was discarded. The stem base sections were then transferred to pre-weighed tubes (5ml, 50 x 15mm) and frozen at -70°C for 1 hour before being freeze dried (-55°C, 10^{-1}Torr) with the lids open for 24 hours. Freeze drying prevented degradation of the samples prior to milling and also allowed the extraction to be carried out in separate tubes minimising the risk of cross-contamination. Once freeze dried the dry weight of the samples was determined and the samples were then stored at 4°C until use. The stem bases were then milled using a Spex 8000 mixer/mill. Two steel ball bearings (diameter 6mm) were added to each tube and the tubes paced in the mixer/mill for 10 minutes. The ball bearings were then removed and the milled material was transferred to 15ml disposable centrifuge tubes.

5.2.9 DNA Extraction

Extraction of the DNA occurred in CTAB buffer by the method described in section 4.2.6. On obtaining the dry DNA pellet it was re-dissolved in TE buffer at a rate of 1.25µl mg^{-1} dry weight of plant material. This DNA stock was then stored at 4°C until use.

5.2.10 Standardisation of the DNA concentration

As the DNA extraction method can have different efficiencies, the DNA concentration was estimated by comparison with DNA standards following gel electrophoresis (10 volts cm^{-1} gel length for 60-90 minutes). Aliquots of DNA were added to bromophenol blue loading dye and run on 1% agarose gels alongside a lambda DNA 50ng μl^{-1} HIND III ladder. Using image analysis the amount of DNA was estimated by comparing the intensity of the DNA bands with a standard curve produced from the HIND III ladder. A pixel scale of 0-255 was used. Sample concentrations were then adjusted to present a uniform concentration.

5.2.11 PCR amplification

Amplification reactions were carried by the method described by Nicholson *et al.* (1996) in volumes of 50µl containing 50ng of DNA. The reaction buffer used was as described in Section 4.2.7. Primers Ty16F/R and TaO5F/R were used to identify W and R-types respectively (Nicholson *et al.*, 1997) (supplied by PE Applied Biosystems).

Amplification was carried as described in Section 4.2.7.

5.2.12 Competitive PCR

Heterologous DNA fragments were used as competitor molecules to prevent heterodimer formation during PCR. These were provided by P. Nicholson, John Innes Centre. The fragments had the same 5' and 3' termini identical to the fungal 'target' primer sites but no internal sequence homology to the 'target' sequence (Nicholson *et al.*, 1997). Competitor DNA templates were added at a rate of 2 fg (W template) and 1.33 fg (R template) per reaction. This concentration allowed approximately equal amplification of both fungal and competitor fragments. If the proportion of the competitor DNA template is too high it will saturate the reaction so much that no fungal DNA will be detected. If it is too low all plants will appear equally infected. Quantification of the fungal DNA was achieved by estimation of the relative degree of amplification of the PCR product and competitor PCR product in each sample. The Ty16F/R competitor fragment was 0.472kb and the TaO5F/R competitor fragment was 0.473kb in size. The primer pairs Ty16F/R gave a product of approximately 1.05kb and TaO5F/R approximately 0.33kb respectively. Following amplification as described in Section 4.2.7 the PCR products of each reaction were separated by electrophoresis through 2% agarose gel using TAE buffer and a 1kb Ladder (10 volts cm^{-1} gel length for 90 minutes). Gels were stained with ethidium bromide (1nm ml^{-1}) and photographed under UV illumination on a 'Gel Doc 1000' system (Bio-Rad). The ratio between the two amplified fragments (from the fungal DNA and the competitor template DNA) was analysed using Molecular Analyst software (Bio-Rad)

which compared the intensity of the DNA bands using a pixel scale of 0-255. Each sample was analysed twice by competitive PCR.

5.3 Results

5.3.1 Germination rate assessment

The conidial germination rates of the strains and hybrids were not significantly different when assessed on MYG agar, with an average germination rate of 99%.

5.3.2 Assessment of disease symptoms on wheat

The development and penetration through the stem base of the disease symptoms described as elliptical shaped lesions were monitored for the two parental strains and four hybrids on a weekly basis. A comparison of the two parental strains revealed that the wheat plants inoculated with the W-type strain 22-20 showed disease symptoms earlier than the wheat plants inoculated with the R-type strain 22-8. The lesions caused by the strain 22-20 initially developed faster, were larger and penetrated further into the stem base than the lesions caused by the strain 22-8. Figure 5.1 shows the progression of disease symptoms of the parental strains 22-20 and 22-8 inoculated on to wheat. Lesions were on average first seen 2-3 weeks after inoculation with strain 22-20 and 3-4 weeks after inoculation with strain 22-8. By the 5^{th} week strain 22-20 had caused large lesions which were seen to penetrate to the second leaf sheath. However the lesions caused by strain 22-8 were smaller and took a week longer to penetrate to the second leaf sheath. By the 8^{th} week of assessment lesion penetration and size were approximately equal between the strains.

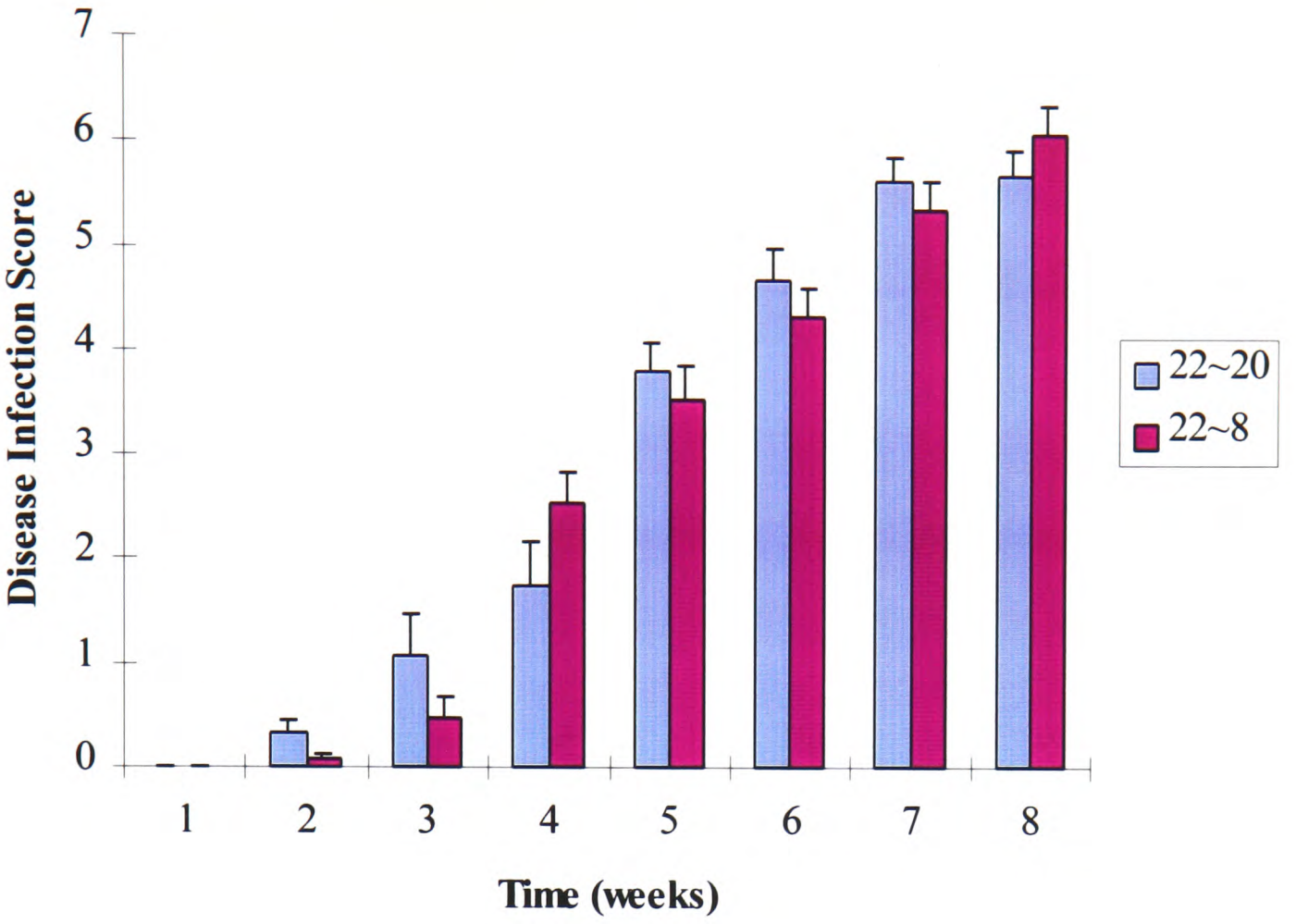


Figure 5.1: Graph showing the development of disease symptoms over 8 weeks of strains 22-20 and 22-8 inoculated on to wheat. Error bars represent the standard error for each mean.

Figure 5.2 shows the progression of disease symptoms of the hybrids P262, O251, C41 and DK inoculated on to wheat. The W x R hybrids P262 and O251 showed reduced disease symptom development with lesions only being present on the coleoptile and first leaf sheath over the 8 week assessment period. The hybrid P262 displayed markings on the coleoptile and first leaf sheath which could have been initial lesion development or natural coleoptile senescence. The hybrid O251 showed markings on the first leaf sheath by the 3rd week and small lesions were present by the 8th week. The plants inoculated with the W x R hybrid C41 showed no disease symptoms over the 8 week assessment. The inter-specific hybrid DK showed disease symptom development similar to that of the R-type strain 22-8.

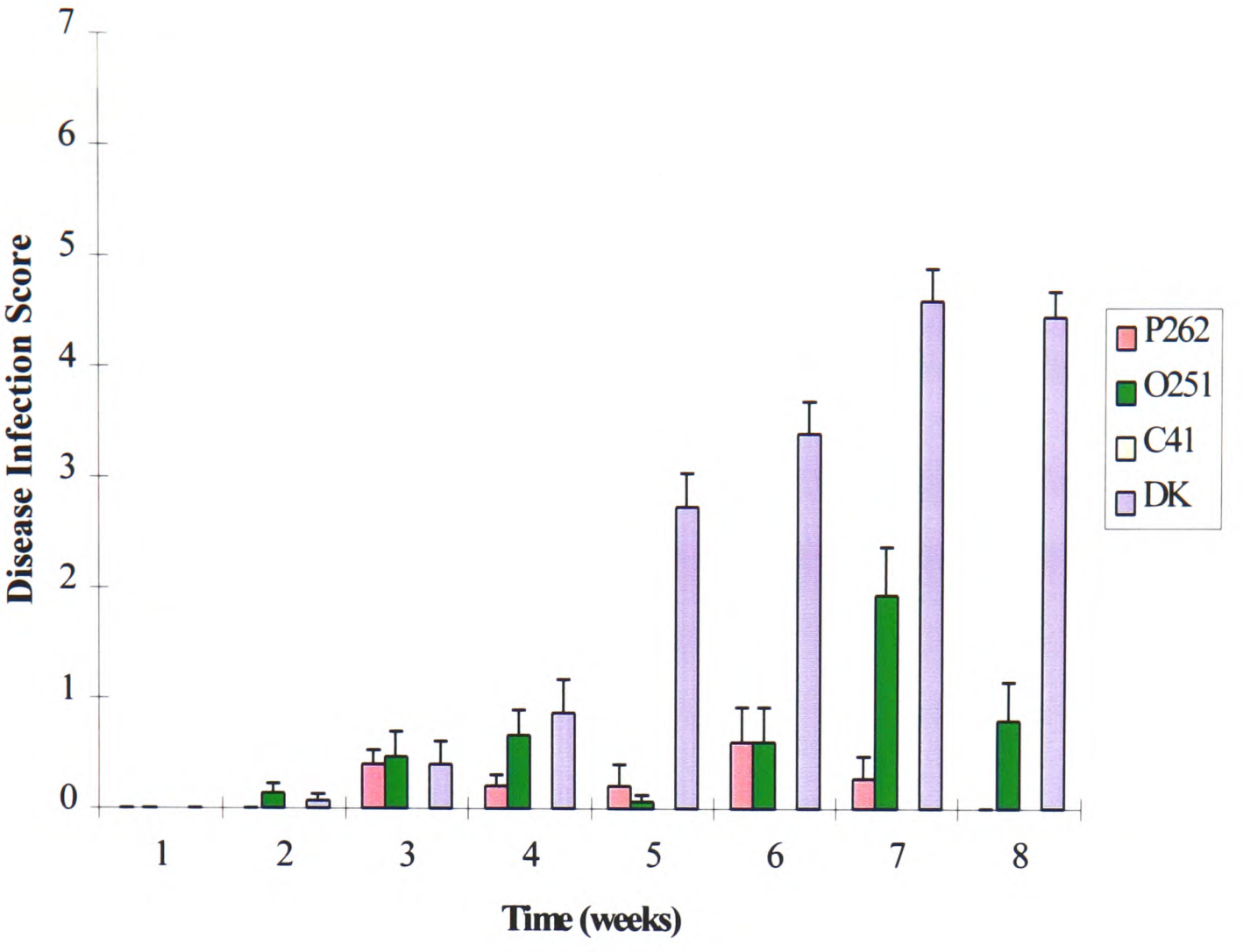


Figure 5.2: Graph showing the development of disease symptoms over 8 weeks of hybrids P262, O251, C41 and DK inoculated on to wheat. Error bars represent the standard error for each mean.

Statistical analysis of the data using the Kruskal-Wallis test for non-parametric data found significant differences ($P<0.05$) at the 2nd and 3rd week assessments; the differences were found between the total data set and the data set when un-inoculated controls were excluded. From the 4th to the 8th week of assessments significant differences ($P<0.05$) were found between strains and hybrids being classed as pathogenic (showing any disease symptoms), the W x R hybrids being less pathogenic than the parental strains. No blocking effect was detected when analysed using a two factor ANOVA with replication, and because the parental strains showed high levels of pathogenicity conditions were suitable for disease development.

5.3.3 Assessment of disease symptoms on rye

A comparison of the disease symptoms on rye plants inoculated with the parental strains 22-20 and 22-8 revealed that lesions caused by the R-type strain 22-8

appeared earlier, were larger and penetrated further into the stem base than the lesions caused by the W-type strain 22-20. This can be seen in Figure 5.3 where disease symptoms appeared by the 2nd week on plants inoculated with strain 22-8, whereas disease symptoms didn't appear until the 3rd week on plants inoculated with strain 22-20. The lesions caused by strain 22-8 penetrated to the third leaf sheath whereas the lesions caused by strain 22-20 remained on the first leaf sheath by the end of the assessment period.

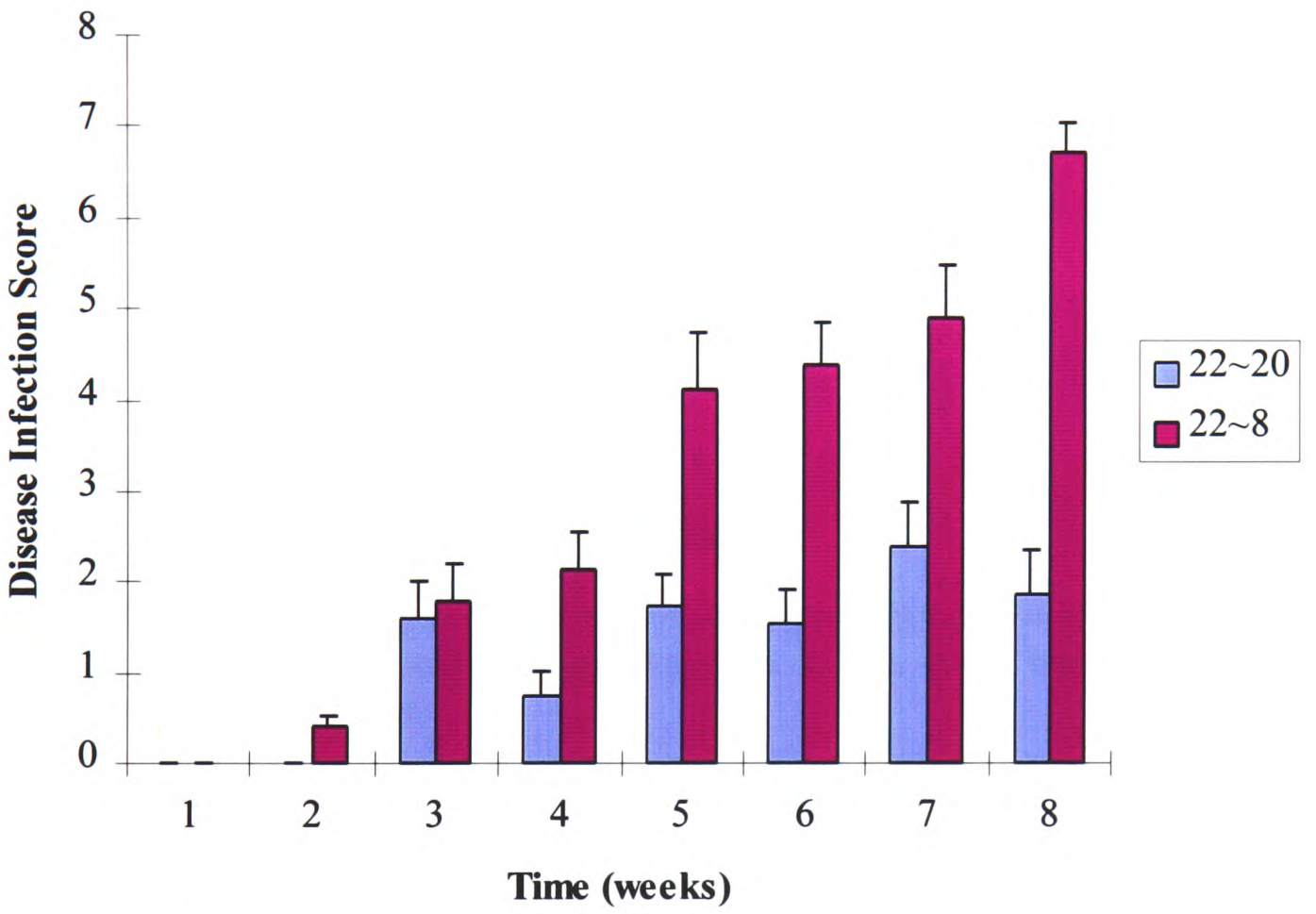


Figure 5.3: Graph showing the development of disease symptoms over 8 weeks of strains 22-20 and 22-8 inoculated on to rye. Error bars represent the standard error for each mean.

Figure 5.4 shows the progression of disease symptoms of the hybrids P262, O251, C41 and DK inoculated on to rye. The W x R hybrids P262 and O251 produced small lesions on the coleoptile and first leaf sheath 1-2 weeks after inoculation. The W x R hybrid C41 produced dark markings only on the coleoptile over the 8 week assessment period. The inter-specific hybrid DK produced lesions only on the coleoptile and the first leaf sheath which appeared 3-4 weeks after inoculation.

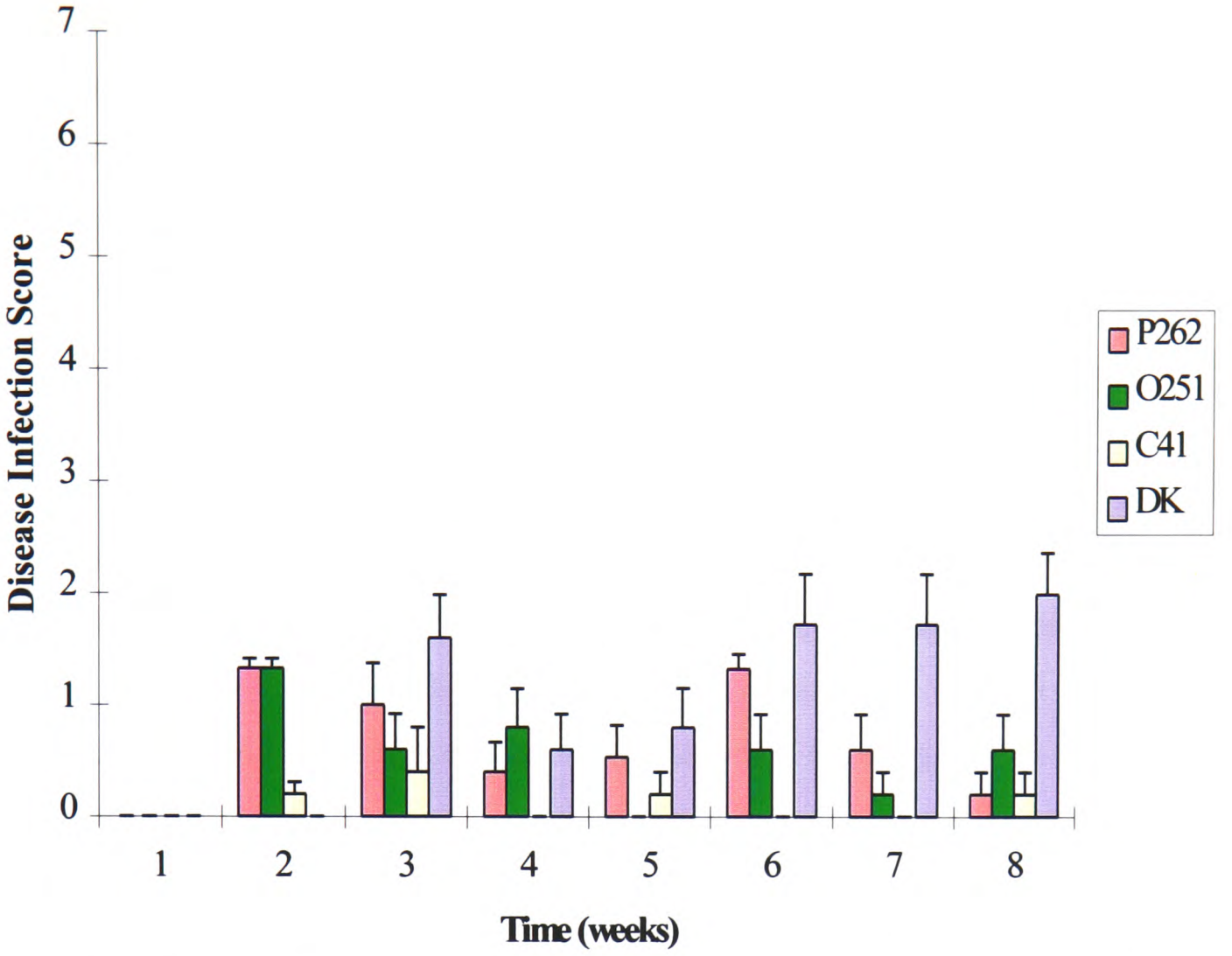


Figure 5.4: Graph showing the development of disease symptoms over 8 weeks of hybrids P262, O251, C41 and DK inoculated on to rye. Error bars represent the standard error for each mean.

Statistical analysis of the data using the Kruskal-Wallis test for non-parametric data found significant differences ($P<0.05$) between the total set of data and when the un-inoculated controls were removed at the 2nd week of assessment. Significant differences ($P<0.05$) were found between the pathogenic strains and hybrids from the 3rd to 8th weeks of assessments. The W x R hybrids were less pathogenic than the parental strains. No blocking effect was detected when analysed using a two factor ANOVA with replication, and as with the experiment using wheat as the host species because the parental strains showed high levels of pathogenicity, conditions were clearly suitable for disease development.

Control plants showed no disease symptoms over the 8 week assessment period.

5.3.4 A comparison of disease symptoms on wheat and rye

A comparison of disease symptom development on wheat and rye by the parental W and R-type strains 22-20 and 22-8 showed that the W-type strain 22-20 produced disease symptoms earlier on wheat than rye. These lesions penetrated further, to the third leaf sheath on wheat whereas they were only seen on the first leaf sheath on rye. In contrast the R-type strain 22-8 produced disease symptoms slightly earlier on rye than wheat but lesion penetration occurred to the same depth in the stem base on both hosts over the assessment period.

The W x R hybrids P262 and O251 produced fewer disease symptoms on wheat and rye compared to their parental strains (22-20 and 22-8), with penetration of the stem base only reaching the first leaf sheath on both hosts. The W x R hybrid C41 produced no disease symptoms on wheat, and only dark markings were seen on rye.

The inter-specific hybrid DK was equal in pathogenicity on wheat compared to its parent R-type strain 22-8. On rye however, disease symptoms were seen later and lacked the same depth of penetration into the stem base.

Figure 5.5 shows the rates of disease symptom penetration over the 8 week assessment of the parental strains and hybrids. The rate is expressed as leaf sheath penetration week^{-1} (the higher the rate then the faster the disease symptoms were seen to occur penetrating through the leaf sheaths of the stem base). The rate was calculated using linear regression on the data of the leaf sheath to which disease symptoms were seen for each sample at each assessment time. It can be seen that the W-type strain 22-20 had a high rate on wheat (0.938) but a low rate (0.276) on rye. The R-type strain 22-8 however had similar rates on both wheat and rye (0.969, 0.946) respectively. The hybrids all had lower rates than the parental strains with the W x R hybrids P262 and O251 and the inter-specific hybrid DK having a higher rate of disease symptom development on wheat compared to rye.

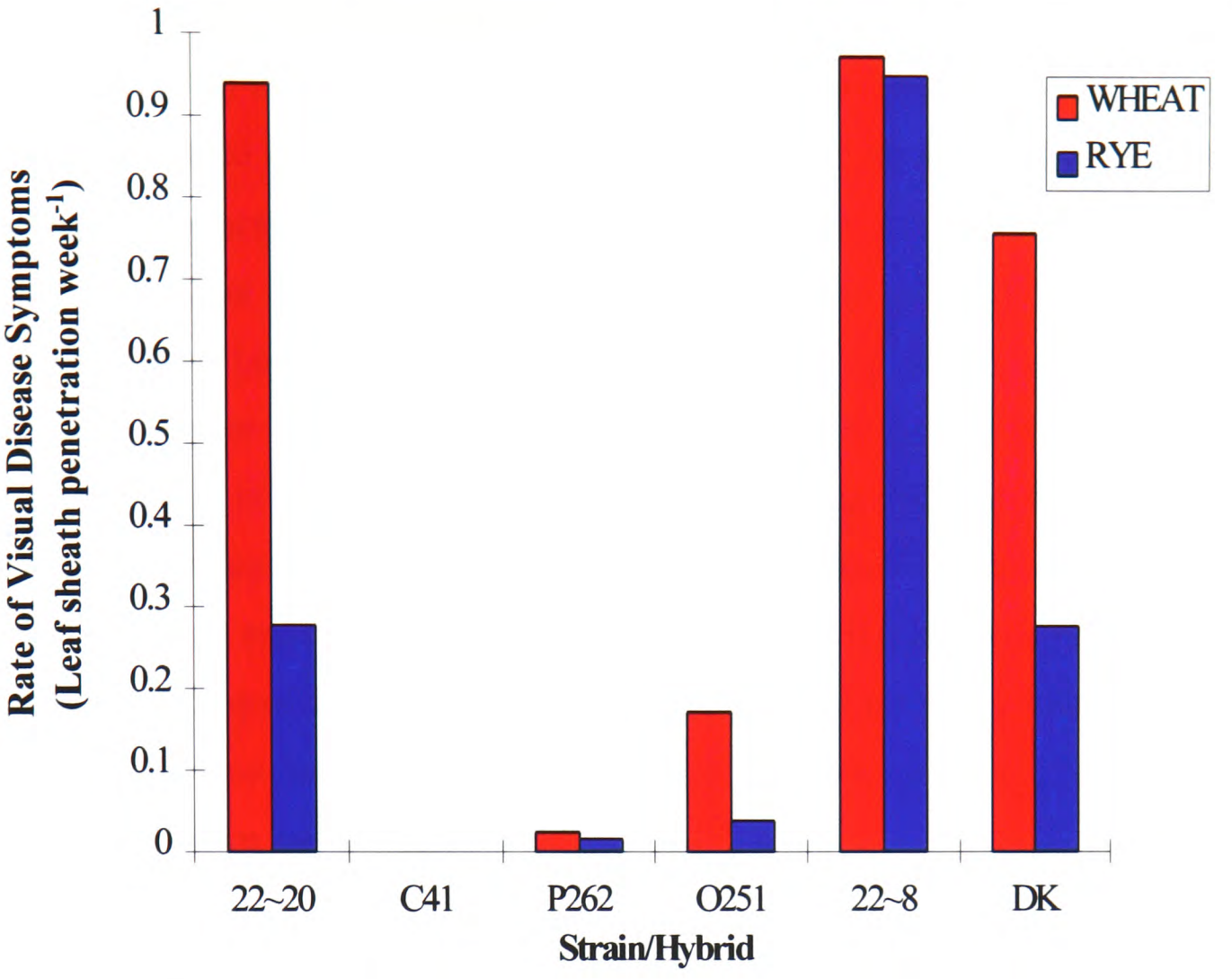


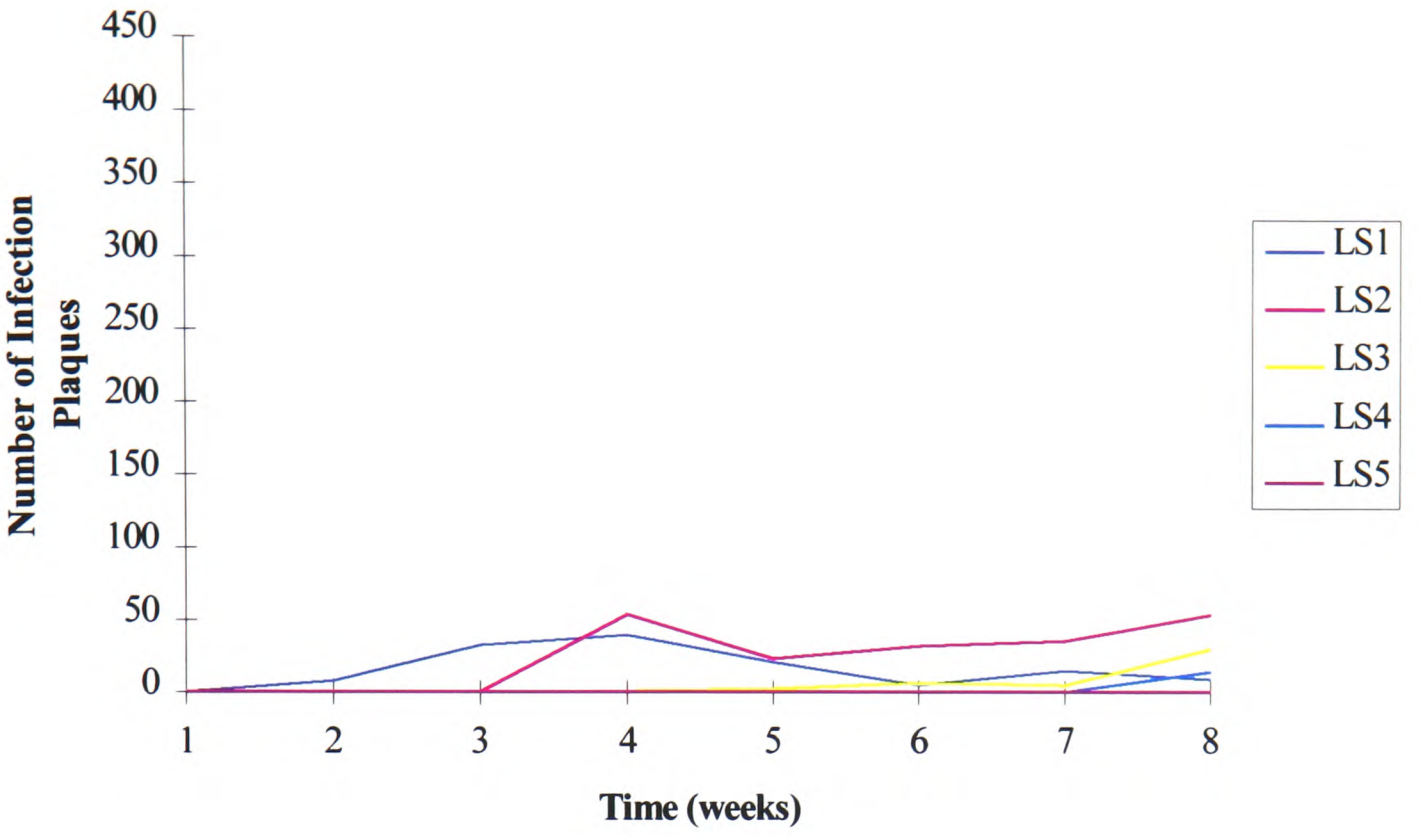
Figure 5.5: Graph showing the rates of disease symptoms of the strains and hybrids inoculated on to wheat and rye.

5.3.5 Microscopic infection structures on wheat

An examination was made for microscopic infection structures on five of the stem bases. This was done by staining all fungal surface structures with a fluorochrome. By the first assessment, 7 days after inoculation, spore germination had occurred in all strains and hybrids. This was shown by single and bipolar germ tubes being produced from the conidial ends. The development of the germ tubes and superficial hyphae was also apparent with there being an indication of anticlinal alignment of the hyphae of the W-type strain 22-20 in the grooves between cells. This was not apparent with the R-type strain 22-8 or any of the hybrids. The cessation of the growth of hyphae and lateral branching in the cells immediately behind the hyphal tip lead to the formation of closely associated swollen parenchyma-like cells which are known as infection plaques. These infection plaques were seen by the 2nd week of assessment in the plants inoculated with the W and R-type strains 22-20 and

22-8 respectively. The infection plaques could be differentiated between the W and R-type strains on the basis of their morphology. W-type plaques were composed of loosely associated cells spreading outwards on the leaf sheath whereas the R-type plaques were discrete and circular, composed of closely associated cells (see Plate 5.2). Colonisation of the leaf sheaths at this stage also included the presence of mycelial plates (see Plates 5.1, 5.2). These mycelial plates were composed of superficial hyphae and runner hyphae which when produced from the W-type strain 22-20 were seen in the anticlinal grooves. The positioning of the hyphae from the R-type strain appeared to be random on the leaf tissue. Infection plaques were seen at the ends of both superficial hyphae and runner hyphae. Hyphal cells produced from the infection plaques either differentiated to enlarge the plaque or became laterally associated to form new runner hyphae from which new infection plaques formed further up or down the leaf sheath.

Figure 5.6 shows the distribution and number of infection plaques seen on each leaf sheath of wheat plants inoculated with strains 22-20 and 22-8 over the 8 week assessment. Both strains produced infection plaques by the 2nd week of assessment. The number of infection plaques produced by the R-type strain 22-8 was much greater than the number produced by the W-type strain 22-20: however strain 22-20 penetrated further into the stem base than strain 22-8. Over the 8 week assessment the W-type strain 22-20 produced more runner hyphae than the R-type strain 22-8. The W-type infection plaques often coalesced (see Plate 5.3) whereas the R-types always remained small and discrete. Secondary sporulation occurred from both W and R-type strains after 4 and 6 weeks respectively.



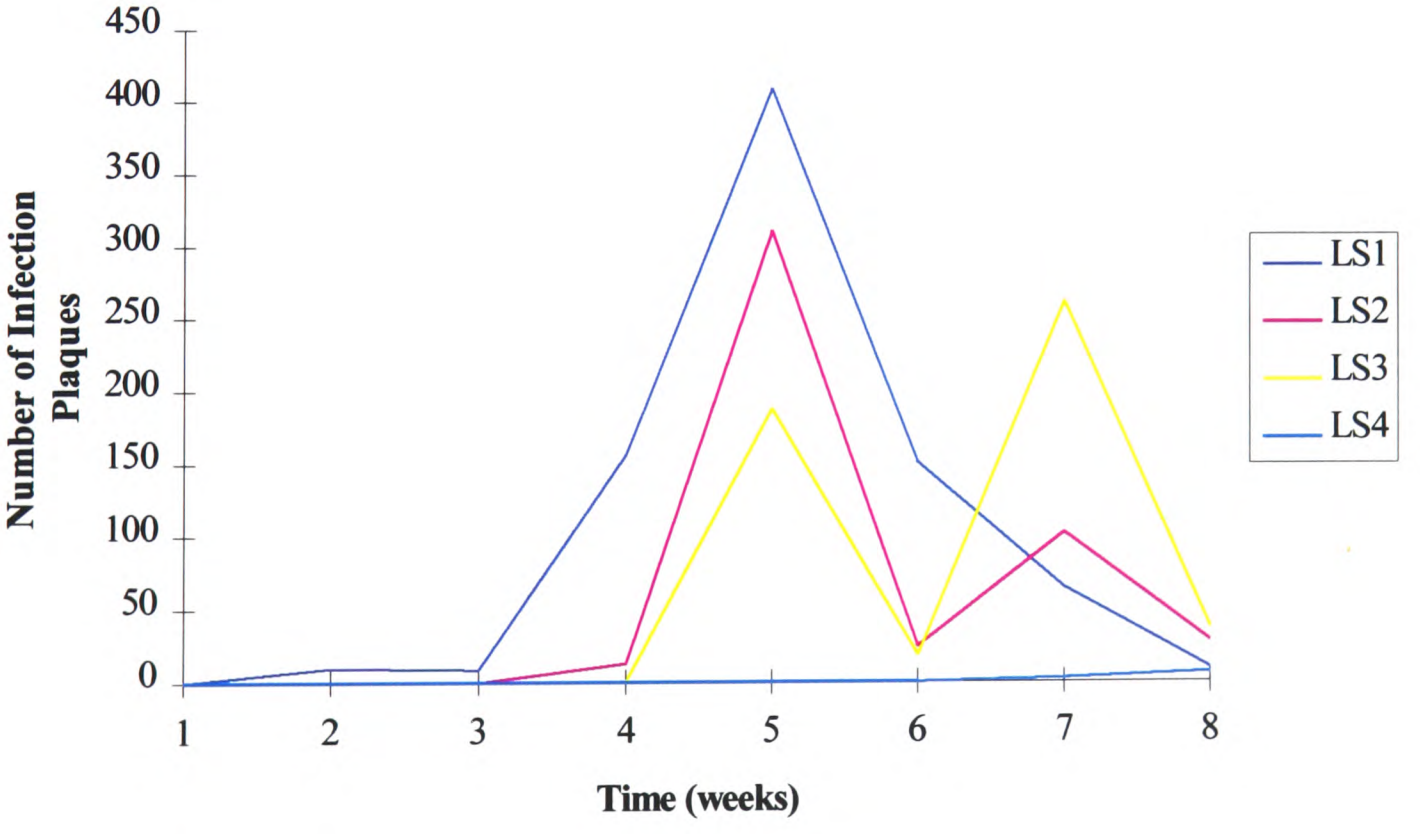


Figure 5.6: Graphs showing the distribution and mean number of infection plaques over a period of 8 weeks of the W and R-types strains 22-20 and 22-8 respectively inoculated on to wheat. LS = Leaf Sheath

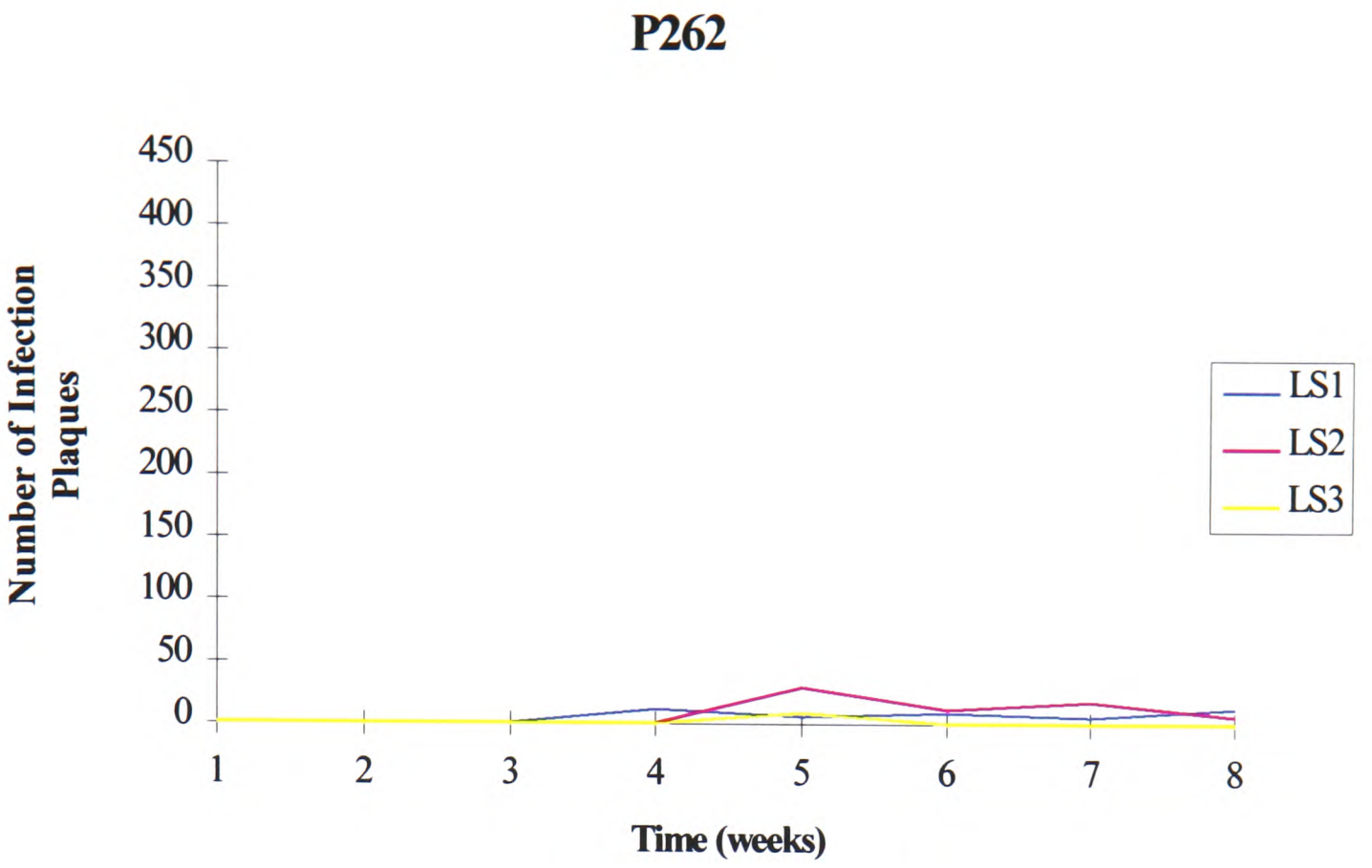


Figure 5.7: Graphs showing the distribution and mean number of infection plaques over a period of 8 weeks of the W x R hybrids P262 and O251 respectively inoculated on to wheat. LS = Leaf Sheath.

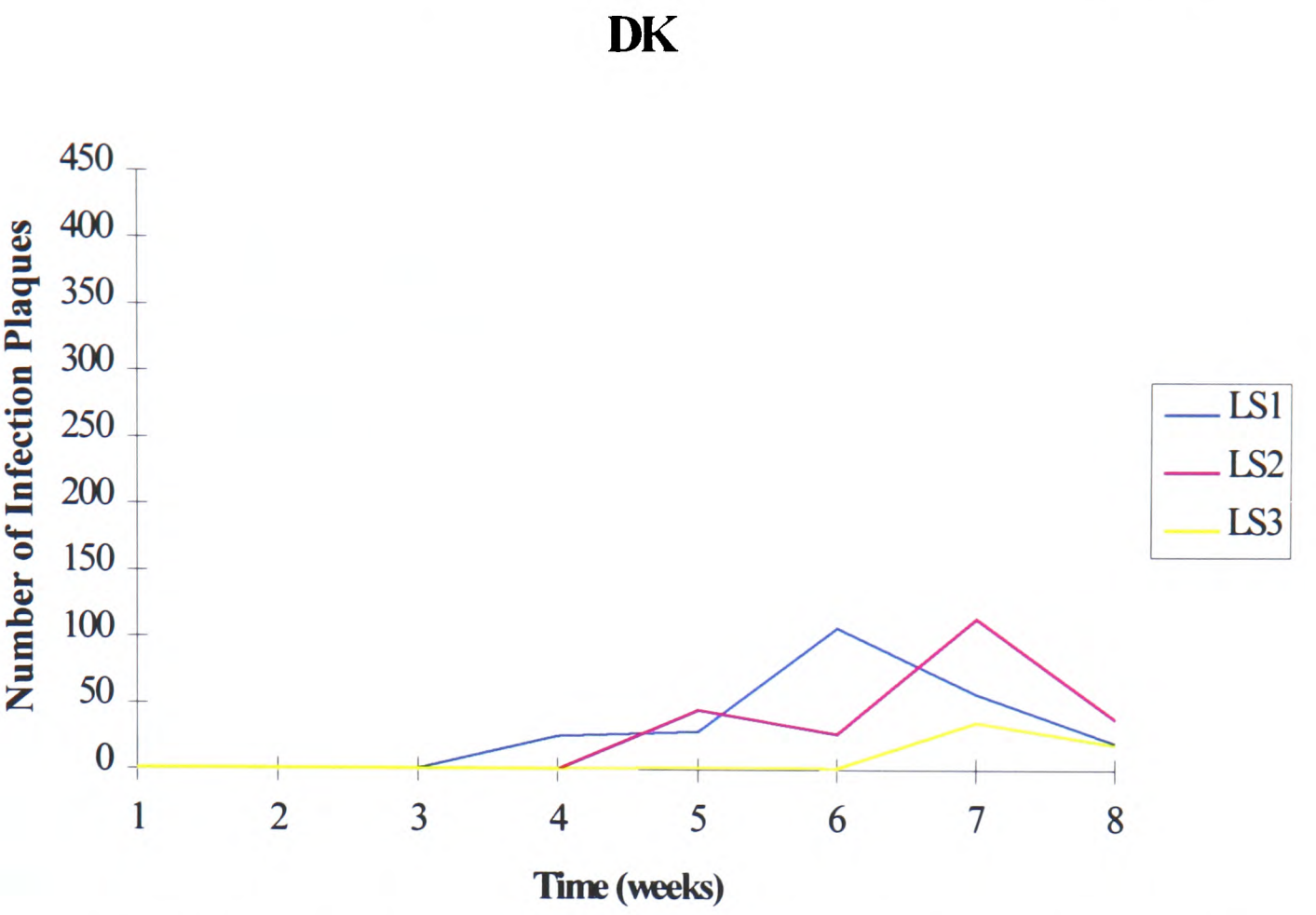


Figure 5.8: Graphs showing the distribution and mean number of infection plaques over a period of 8 weeks of the inter-specific hybrid DK inoculated on to wheat. LS = Leaf Sheath.

Figure 5.7 shows the distribution and number of infection plaques seen on each leaf sheath of wheat plants inoculated with W x R hybrids P262 and O251. Both P262 and O251 produced fewer infection plaques and did not penetrate as far compared to the parental W and R-parental strains. Infection plaques were seen by the 3rd week of assessment and these only penetrated to the third and second leaf sheaths respectively. The infection plaques were immature (only a few swollen cells) and of a W-type. Runner hyphae were rarely seen.

The inter-specific hybrid C41 was found only to germinate and the spore numbers appeared to be reduced compared to the other strains and hybrids; this suggests a lack of adhesion to the coleoptile. Slightly swollen cells were seen at the end of the germ tubes but no infection plaques.

Figure 5.8 shows the distribution and number of infection plaques seen on each leaf sheath of wheat plants inoculated with the inter-specific hybrid DK. The hybrid DK was seen to penetrate to the third leaf sheath over a similar time scale to the R-type strain 22-8. Infection plaques were of an R-type and their numbers were approximately a third that of the R-type strain 22-8. Runner hyphae were present but in fewer numbers than seen with the R-type strain 22-8.

No secondary sporulation occurred with any of the W x R or inter-specific hybrids on wheat.

In assessments made of infection plaque numbers produced by the parental strains and hybrids on both wheat and rye, it was found that generally plaque numbers peaked 1-2 weeks after they were first seen on each leaf sheath. Infection plaque numbers then tended to decrease over the remaining assessment period. This may be due to the plaques coalescing and being counted as 1 instead of 3. The infection plaques may have fallen off during handling and staining especially if necrosis had occurred, or if mucilage which may aid in attachment to the cells had dried out causing the infection plaques to fall off.

5.3.6 Microscopic infection structures on rye

The form of the infection structures on rye was found to be the same as those on wheat when inoculated with the parental W and R-type strains 22-20 and 22-8. The W- type strain produced characteristic large coalescing W-type infection plaques and the R-type strain produced characteristic small discrete R-type plaques as described previously. Spore germination and infection plaque production occurred by the first week of assessment on rye plants inoculated with W and R-type strains 22-20 and 22-8. Figure 5.9 shows the distribution and numbers of infection plaques on the rye plants inoculated with strains 22-20 and 22-8 over the 8 weeks of assessment. It can be seen that the R-type strain 22-8 produced more infection plaques earlier than the W-type strain 22-20. The R-type strain 22-8 also penetrated further into the stem base, to the fourth leaf sheath compared to the W-type strain which penetrated only to

the third leaf sheath. Both strains produced runner hyphae and showed anticlinal alignment of hyphae and infection plaques. Secondary sporulation occurred after 4 weeks by the W-type strain 22-20 and after 6 weeks by the R-type strain 22-8.

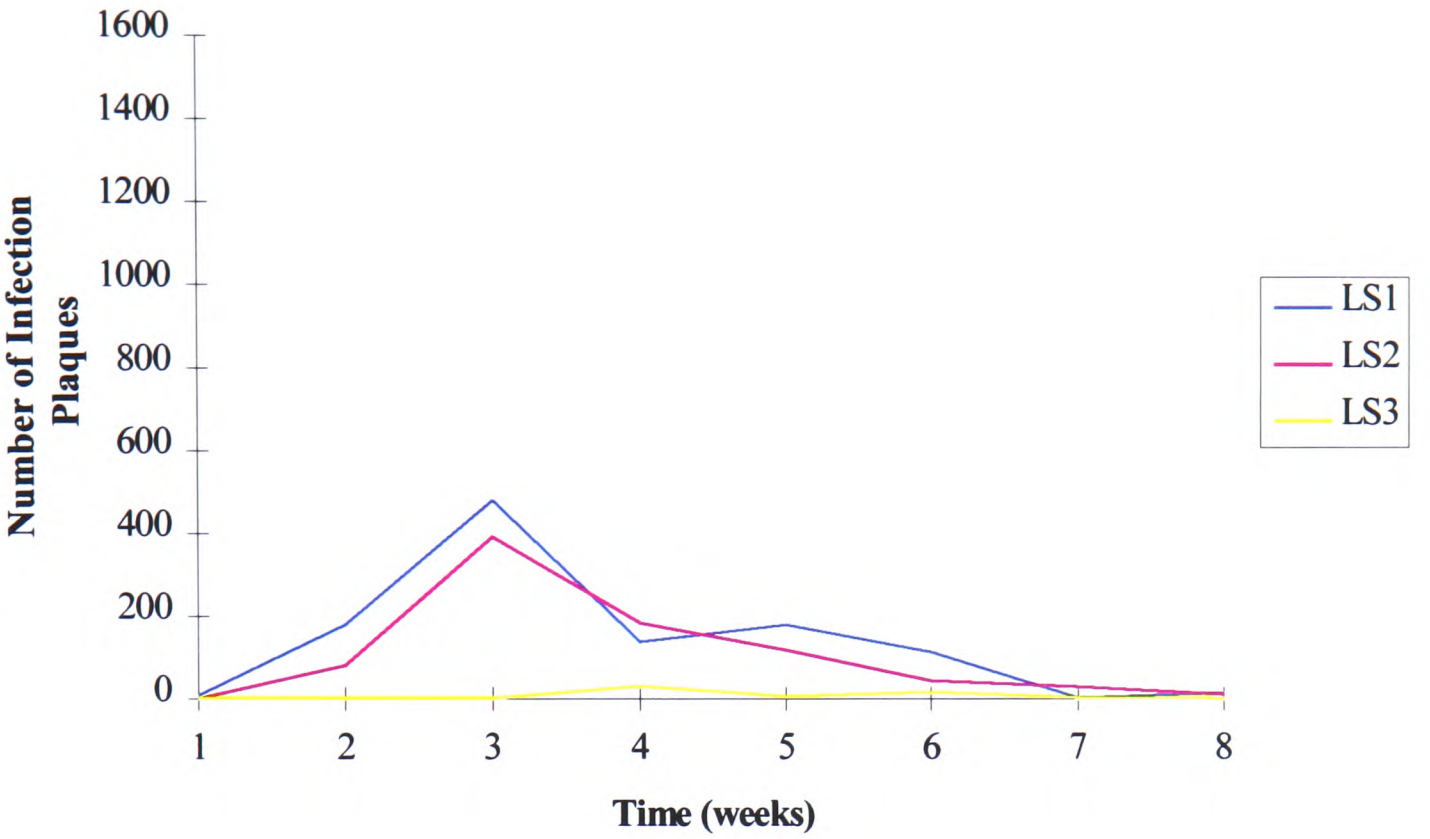


Figure 5.9: Graphs showing the distribution and mean number of infection plaques over a period of 8 weeks of the W and R-types strains 22-20 and 22-8 respectively inoculated on to rye. LS = Leaf Sheath.

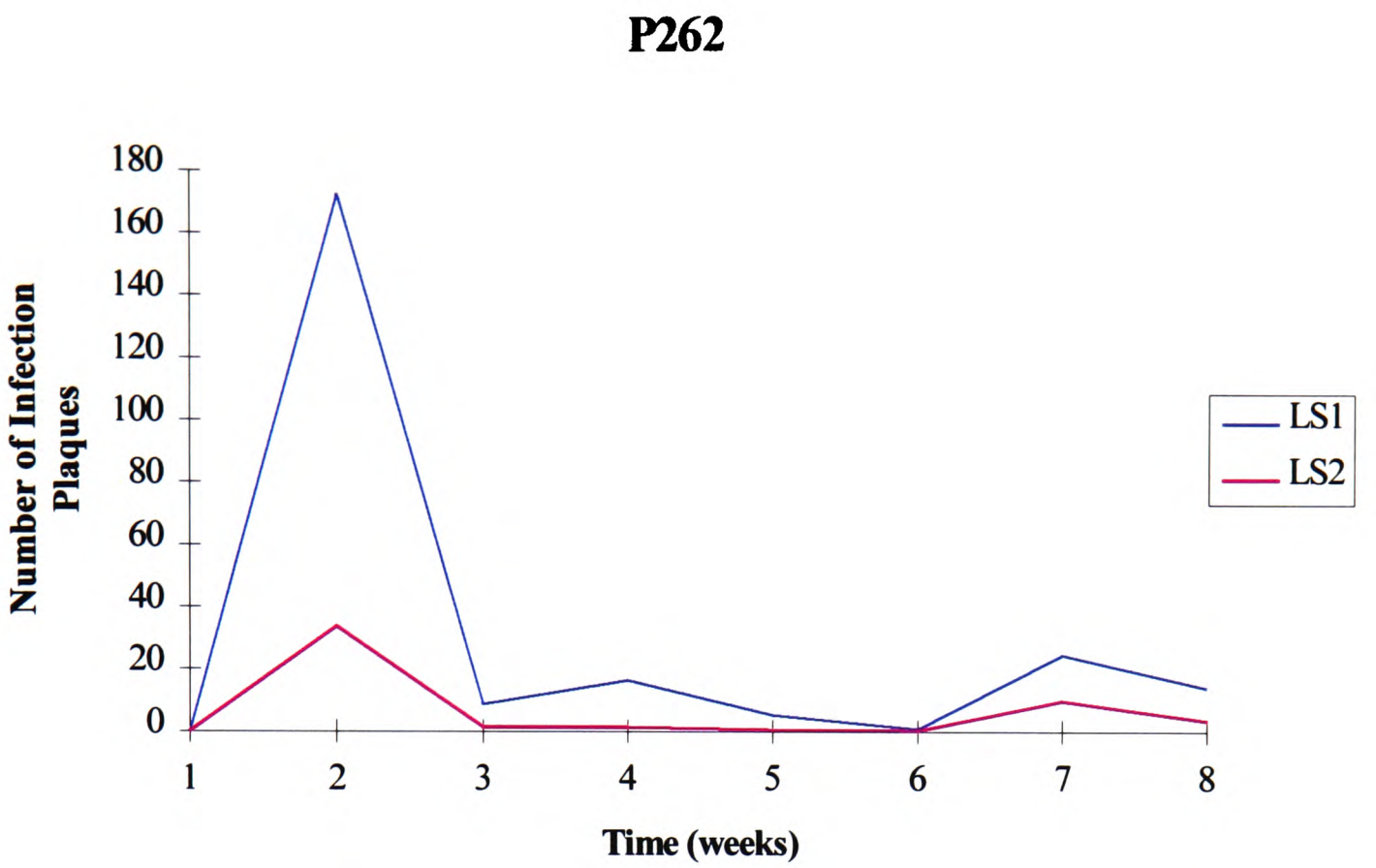
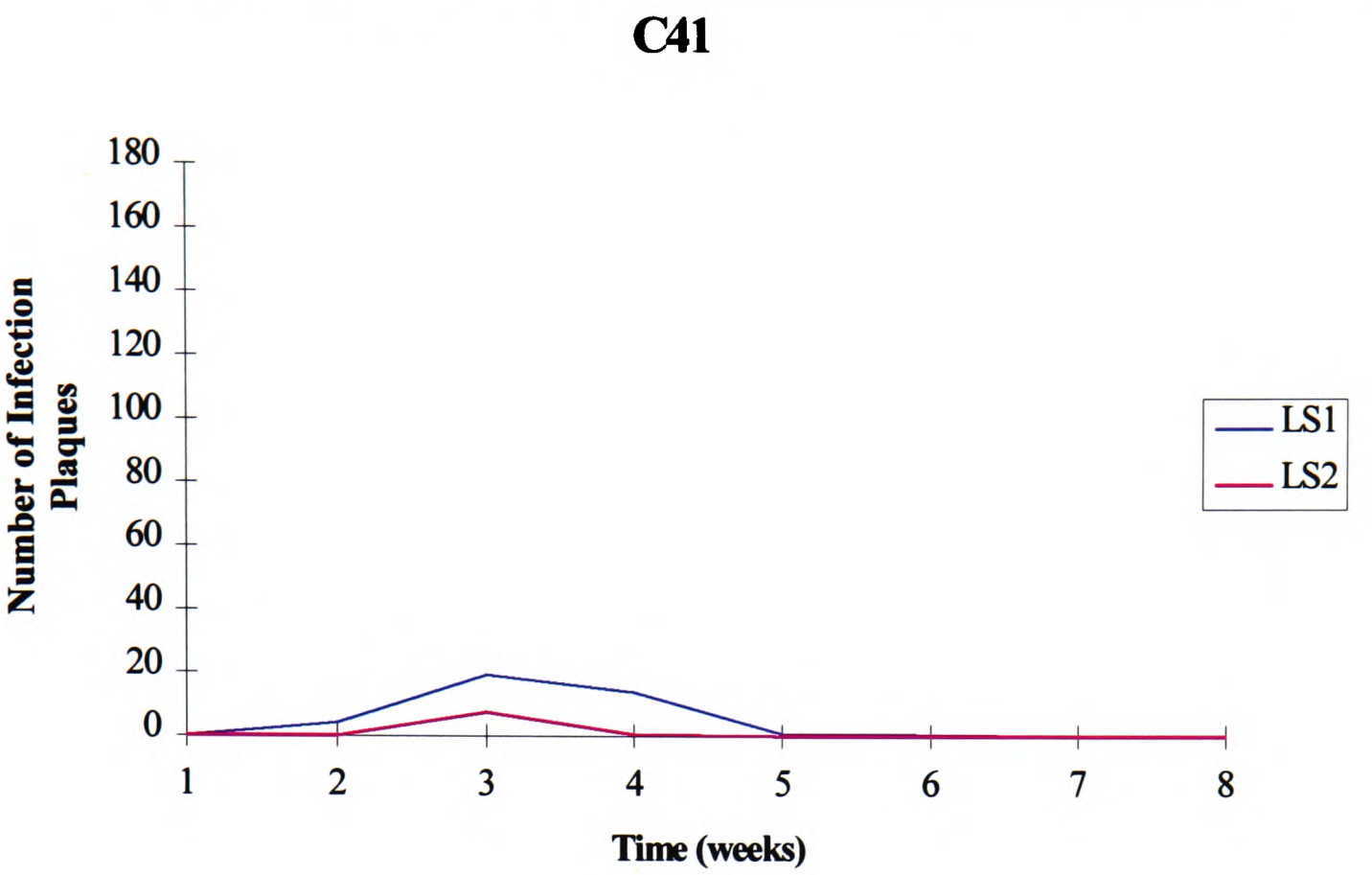


Figure 5.10: Graphs showing the distribution and mean number of infection plaques over a period of 8 weeks of the W x R hybrids P262 and O251 respectively inoculated on to rye. LS = Leaf Sheath.



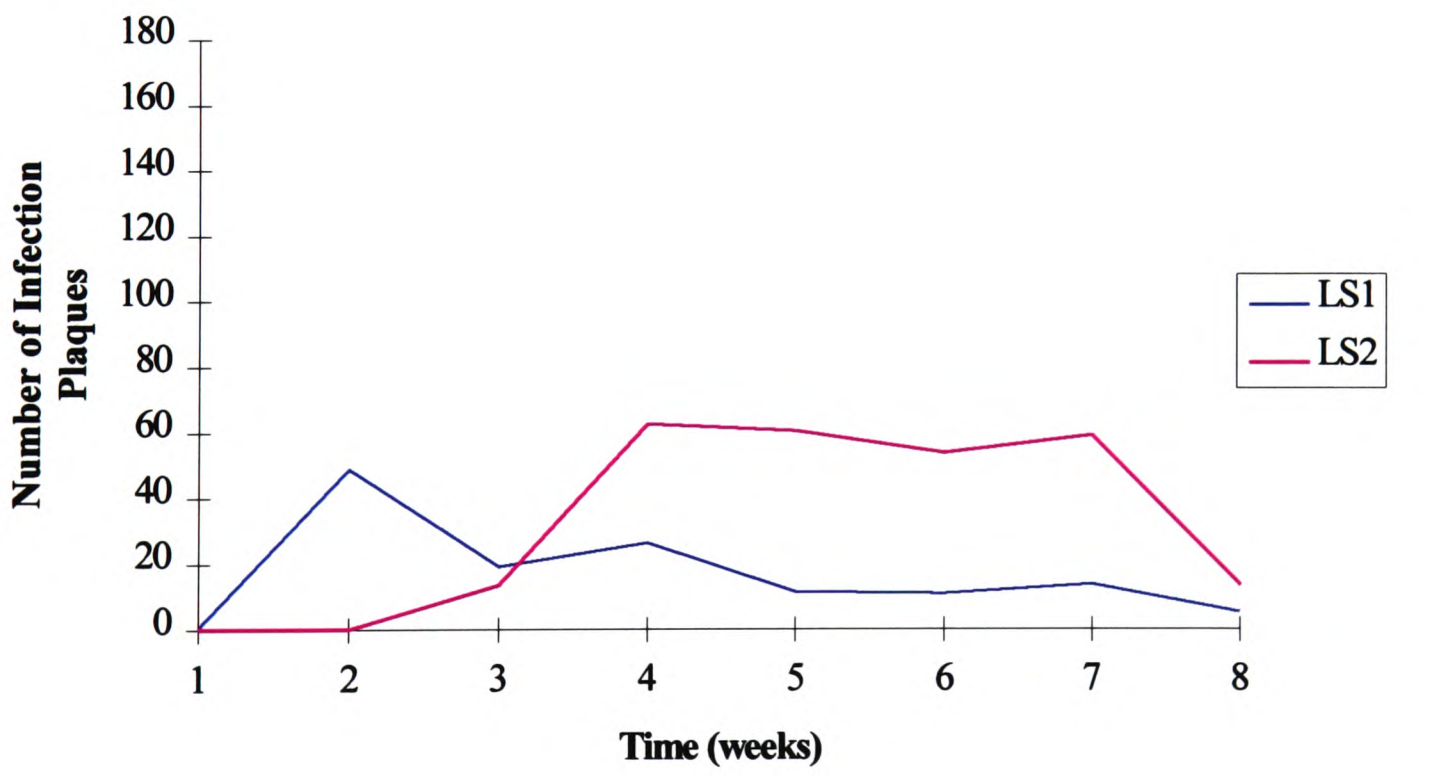


Figure 5.11: Graphs showing the distribution and mean number of infection plaques over a period of 8 weeks of the W x R hybrid C41 and inter-specific hybrid DK respectively inoculated on to rye. LS = Leaf Sheath.

Figure 5.10 shows the distribution and number of infection plaques seen on each leaf sheath of rye plants inoculated with the W x R hybrids P262 and O251. The hybrids P262 and O251 produced infection plaques by the first week. However, penetration by both hybrids never occurred further than the second leaf sheath. Both hybrids produced infection plaques of a W-type. These were seen to enlarge over time, with hybrid P262 producing more plaques than hybrid O251. Some anticlinal alignment of the runner hyphae was seen. However generally were sparse.

Figure 5.11 shows the distribution and number of infection plaques seen on each leaf sheath of rye plants inoculated with the W x R hybrid C41. This hybrid produced many swollen cells at the end of the hyphae, but where infection plaques were seen they were few in number and resembled immature W-types. The furthest the infection plaques were seen to penetrate was to the 2nd leaf sheath. Runner hyphae were present but few in number.

The inter-specific hybrid DK produced infection plaques by the first week of assessment, penetration of these plaques occurred only to the second leaf sheath over the 8 week period. Their number and distribution can be seen in Figure 5.11. The plaques resembled R-type ones and runner hyphae were present which showed some anticlinal alignment.

No secondary sporulation occurred with any of the W x R or inter-specific hybrids on rye.

Control plants showed no evidence of microscopic infection structures produced by *P. herpotrichoides* over the 8 week assessment period.

5.3.7 A comparison of microscopic infection structures on wheat and rye

A comparison of microscopic infection structures on wheat and rye by the parental strains 22-20 and 22-8 found that strain 22-20 produced W-type plaques, runner

hyphae, anticlinal alignment and showed secondary sporulation on both hosts. The W-type strain 22-20 produced more plaques on rye than wheat and penetration into the stem base occurred earlier on rye than wheat. However, by the end of the assessment period the depth of penetration was greater on wheat to the fifth leaf sheath compared to rye where it reached the 3rd leaf sheath.

The R-type strain 22-8 produced R-type plaques and runner hyphae on both hosts. Anticlinal alignment occurred only on rye but secondary sporulation occurred on both. The number of infection plaques was greater on rye than wheat and the penetration into the stem base occurred earlier on rye compared to wheat but by the end of the assessment period the depth of penetration into the stem base was the same for both.

The W x R hybrids P262 and O251 produced W-type plaques on both hosts but these were however often immature. For both hybrids, infection plaques were produced in greater numbers on rye compared to wheat and penetrated the leaf sheaths earlier on the former. Colonisation of the host by the hybrids was reduced probably because of a lack of runner hyphae and no secondary sporulation, although secondary sporulation is not thought to contribute directly to disease symptom development (Daniels *et al.*, 1991).

The W x R hybrid C41 produced microscopic infection structures only on rye and these were immature W-type plaques which were only seen to the second leaf sheath.

The inter-specific hybrid DK produced R-type infection plaques and runner hyphae on both hosts. Some anticlinal alignment was seen to occur on rye. Colonisation of rye occurred earlier than wheat but the depth of penetration had progressed further on wheat by the end of the assessment period. Infection plaque numbers were slightly less on rye.

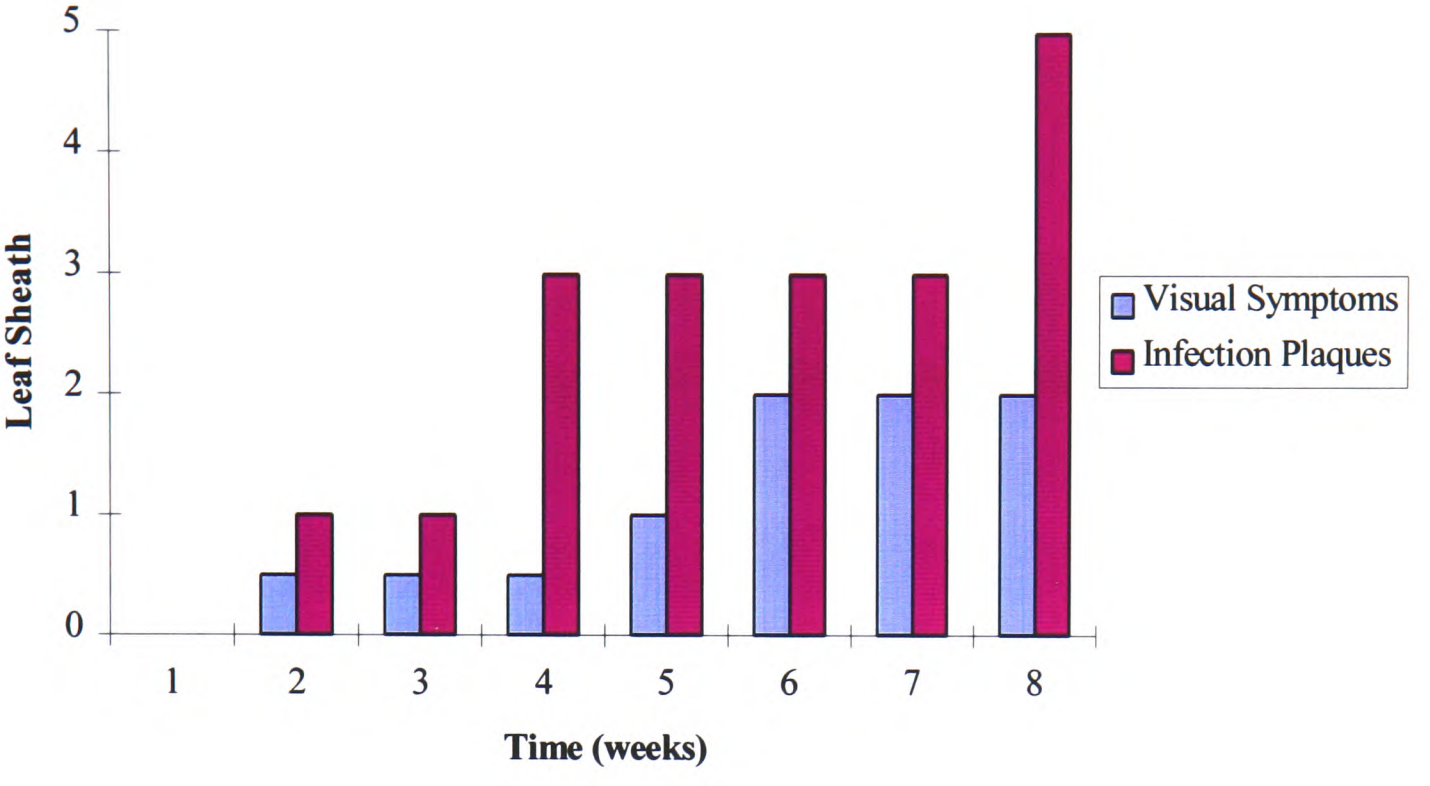
The generalised logistic curve for growth modelling gave the best line fit when an examination was made of the furthest leaf sheath to which infection plaques were seen at each assessment time. The data was transformed using $\sqrt{(X+0.5)}$ to normalise it and analysed using Genstat 5. The parental strains 22-8 and 22-20 and hybrids P262, O251 and DK showed an increased lag phase of 1-2 weeks in the time taken to produce infection plaques on the first leaf sheath on wheat compared to rye. The other parameters were not comparable due to the parental strain or hybrids not progressing through the stem base and also because the assessment was only performed over 8 weeks which may not have been a long enough time for all the strains and hybrids to reach a stationary phase of growth. Examination of Figures 5.6-5.11 of strains 22-20, 22-8, W x R hybrids P262, O251 and C41 and inter-specific hybrid DK indicates that progression of microscopic infection structures through the leaf sheaths after the lag phase occurs at a similar rate on wheat and rye.

5.3.8 A comparison of disease symptoms and microscopic infection structures on wheat and rye

Comparing disease symptoms with microscopic infection structures it is seen that microscopic infection structures such as infection plaques were always found at least 1 leaf sheath in advance of the disease symptoms. Figures 5.12 and 5.13 show the furthest leaf sheaths to which disease symptoms and infection plaques were found on wheat and rye plants inoculated with the W and R-type strains 22-20 and 22-8 respectively. It can be seen that although the W-type strain 22-20 was unable to cause disease symptoms beyond the 1st leaf sheath on rye plants, infection plaques were present on the 3rd leaf sheath.

Similarly the W x R and inter-specific hybrids had microscopic infection structures in advance of any disease symptoms. Where examination of lesions was made the infection plaques were generally found at the necrotic margin of the lesion.

WHEAT



RYE

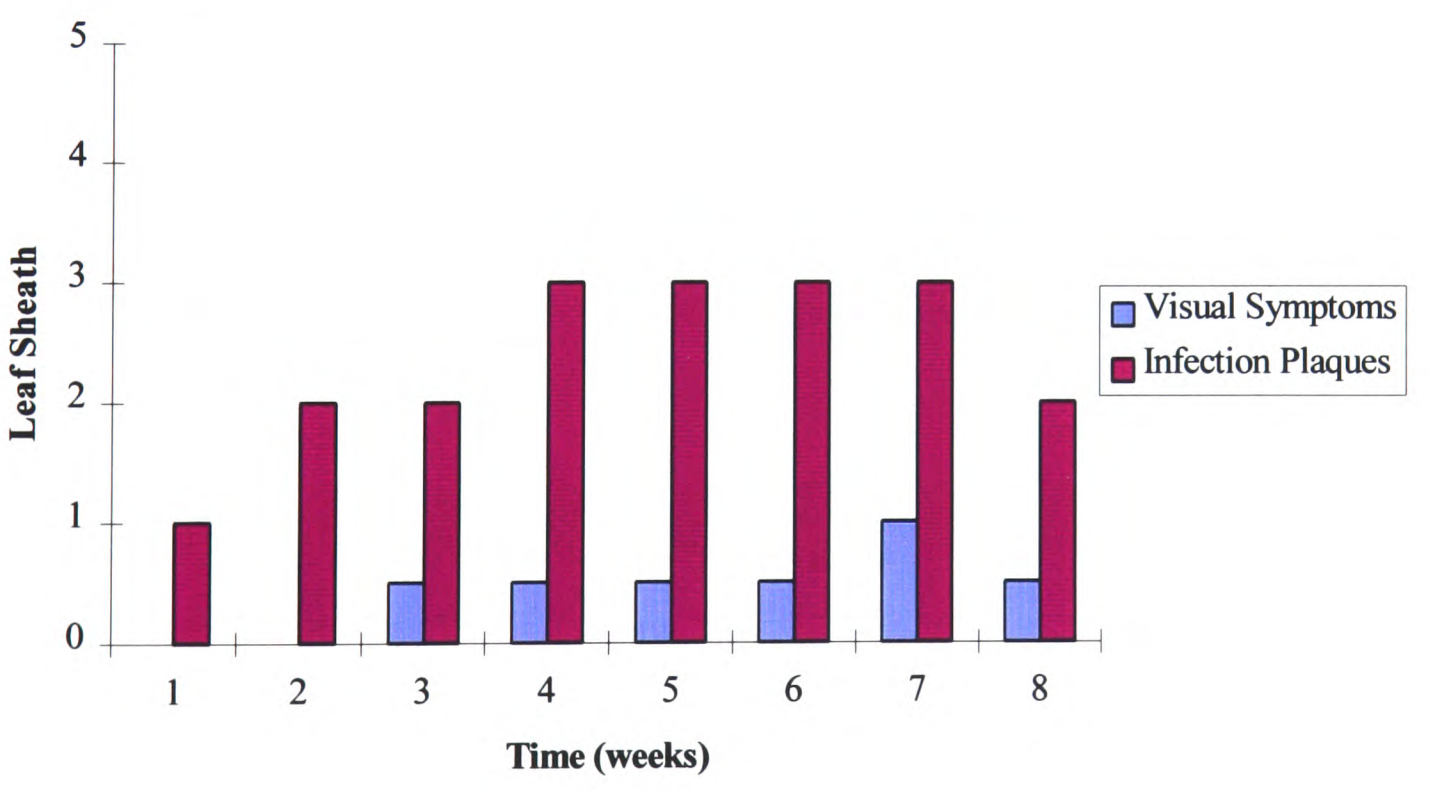


Figure 5.12: Graphs showing the furthest leaf sheaths on which disease symptoms and infection plaques were found over the assessment period on wheat and rye plants inoculated with the W-type strain 22-20. A score of 0.5 = coleoptile.

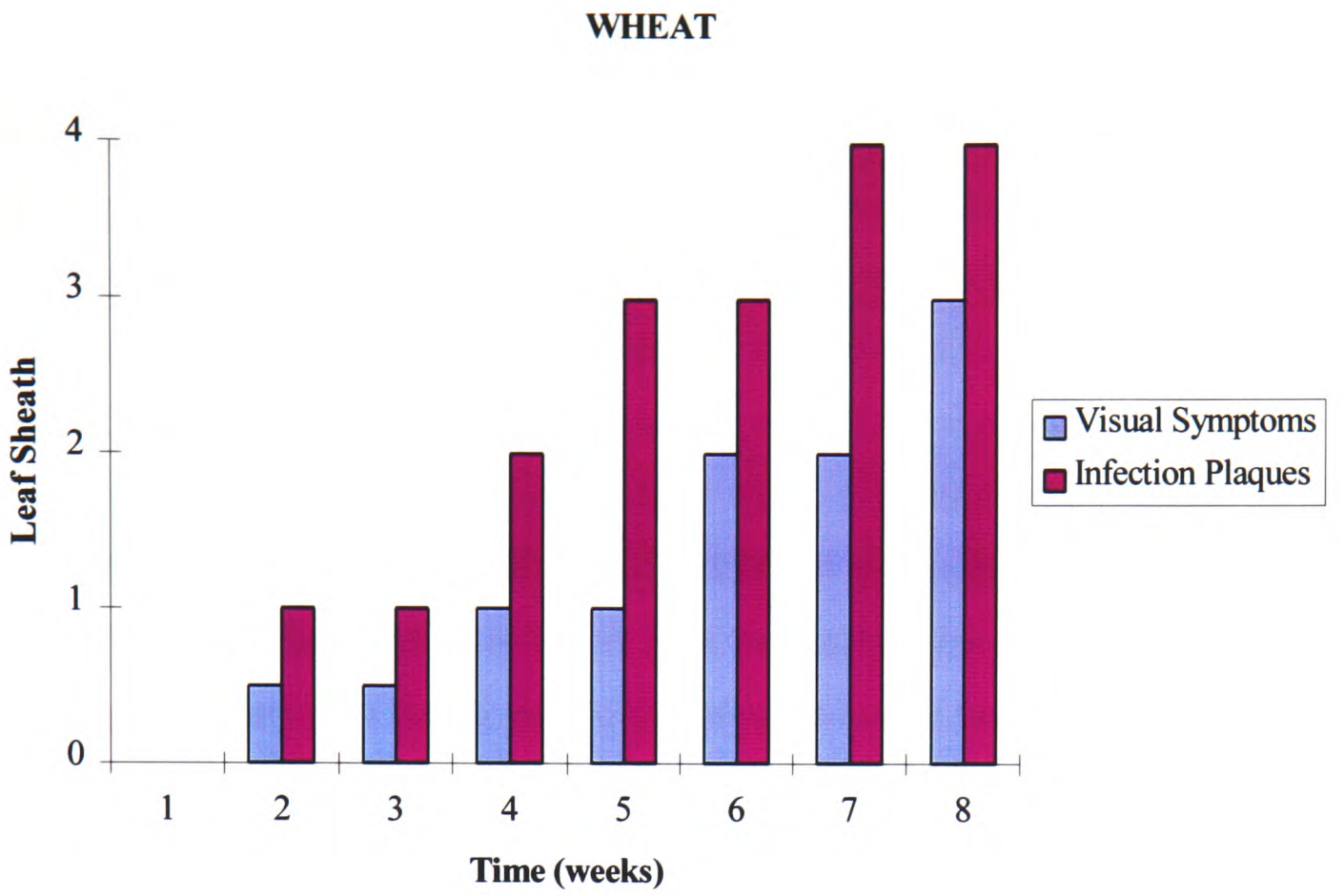


Figure 5.13: Graphs showing the furthest leaf sheaths on which disease symptoms and infection plaques were found over the assessment period on wheat and rye plants inoculated with the R-type strain 22-8. A score of 0.5 = coleoptile.

5.3.9 Quantification of fungal DNA by competitive PCR

Competitive PCR was carried out using primers Ty16F/R and Ta05F/R in the presence of the selected amount of the competitor DNA template on 2 samples consisting of 5 stem bases per sample. Each sample was analysed twice. The ratio of the fungal amplification product to the competitor product was proportional to the amount of fungal DNA present in the sample (see Plate 5.4). Thus the amount of fungal DNA per 50ng total DNA (plant and fungal DNA) is expressed as a PCR ratio. The ratio was obtained using image analysis which determined the intensity of the amplified DNA products. A pixel scale of 0-255 was used and background readings were subtracted. The data distribution was normalised by transforming it with $\sqrt{(X+1)}$. A PCR ratio of 1 (using transformed data) indicated that no fungal DNA was present (the higher the PCR ratio the more fungal DNA present).

The control samples were amplified using both primer and competitor sets. No fungal fragment was detected identifying either W or R-types in these samples indicating that no cross contamination had occurred. The amplification of the competitor fragment confirmed that the PCR protocol was working.

Quantification of the parental W-type strain 22-20 and W x R hybrids P262, O251 and C41 was carried out using the primer pair Ty16F/R and the parental R-type strain 22-8 and the inter-specific hybrid DK using the primer pair Ta05F/R and their respective competitor DNA templates. Detection of the W and R fungal PCR products using these primer pairs in the parental strains and hybrids has previously been described in chapter 4.

5.3.10 Quantification of fungal DNA from the parental strains 22-20 and 22-8 inoculated on to wheat and rye

Quantification of the W-type strain 22-20 showed that it was present in equal quantities in both wheat and rye. Analysis of the transformed data using single factor ANOVAs and two factor ANOVAs with replication found no significant differences

between the PCR ratios at any of the assessment periods or between the two hosts at these periods. Figure 5.14 shows the mean PCR ratios (transformed data) obtained from wheat and rye. For strain 22-20 it can be seen that the amount of fungal DNA varied but did not increase over time in either host.

The amount of fungal DNA was significantly ($P<0.05$) greater in the rye plants compared to the wheat plants inoculated with the R-type strain 22-8 over all of the assessments except those assessments made at weeks 6 and 7. Significant differences ($P<0.05$) were also found between assessment periods for both wheat and rye. From Figure 5.14 it can be seen that the amount of DNA of strain 22-8 increased over the assessment period from both wheat and rye.

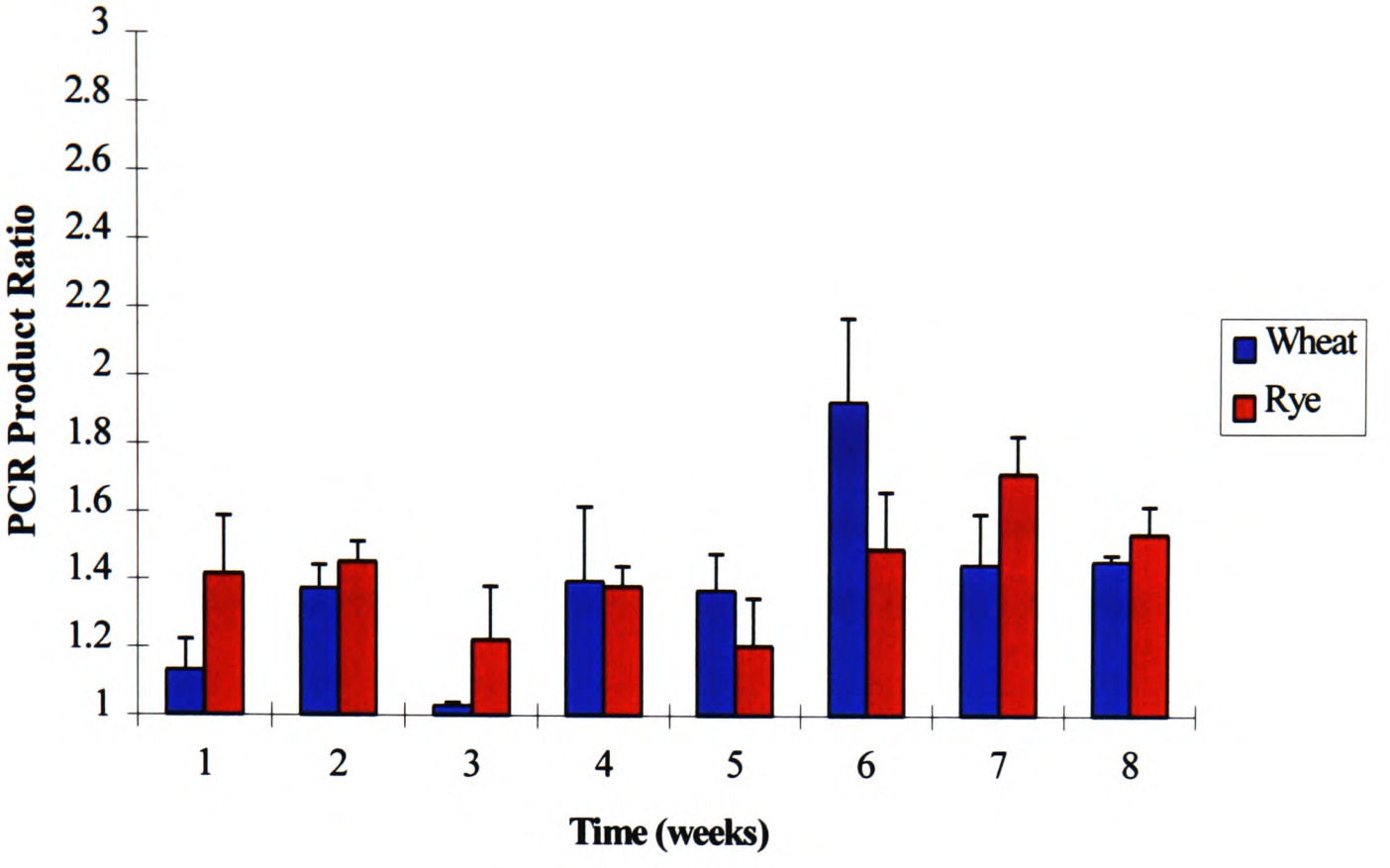


Figure 5.14: Graphs showing the mean amount of fungal DNA expressed as a PCR ratio in wheat and rye plants inoculated with strains 22-20 and 22-8 over an infection period of 8 weeks. Error bars represent the standard error for each mean.

5.3.11 Quantification of fungal DNA from the W x R hybrids and inter-specific hybrid inoculated on to wheat and rye

All the W x R hybrids generally gave higher PCR ratios on rye than wheat. Significant differences ($P<0.05$) were found throughout the 8 weeks assessments both between wheat and rye and between assessment periods but these appear to be random for each of the hybrids except DK where the quantity of DNA increased over time on both wheat and rye.

Figures 5.15 and 5.16 show the PCR ratios from wheat and rye plants inoculated with hybrids P262, O251 and C41. It can be seen that the quantity of fungal DNA fluctuated over the 8 weeks with no pattern being seen for all 3 hybrids.

Quantification of the DNA from plants inoculated with the inter-specific hybrid DK show that it was significantly ($P<0.05$) present in greater amounts on rye than wheat from the 6th assessment week onwards (see Figure 5.16). The quantity of DNA increased in both wheat and rye plants over the 8th week assessment period.

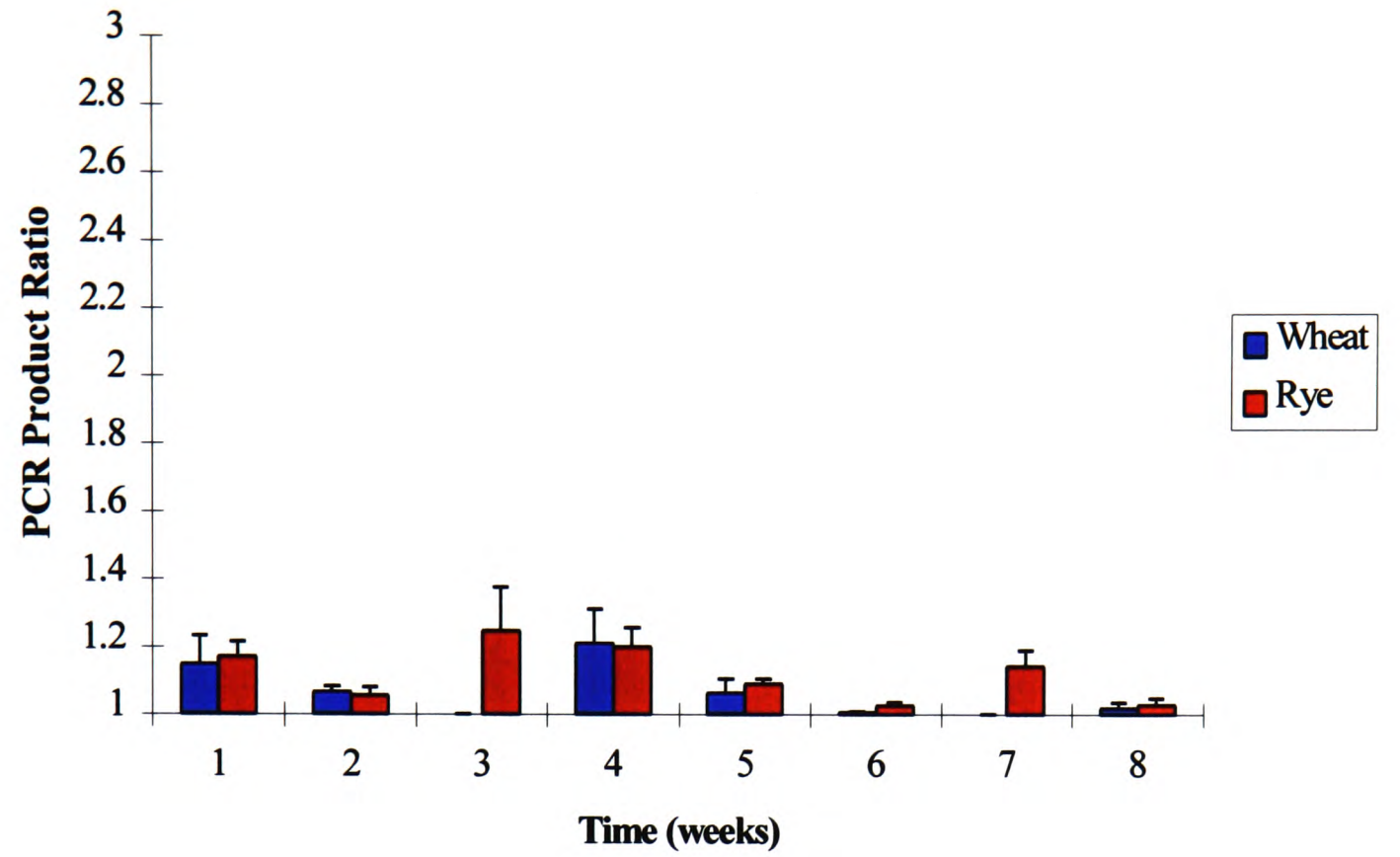


Figure 5.15: Graph showing the mean amount of fungal DNA expressed as a PCR ratio in wheat and rye plants inoculated with W x R hybrids P262 and O251 over an infection period of 8 weeks. Error bars represent the standard error for each mean.

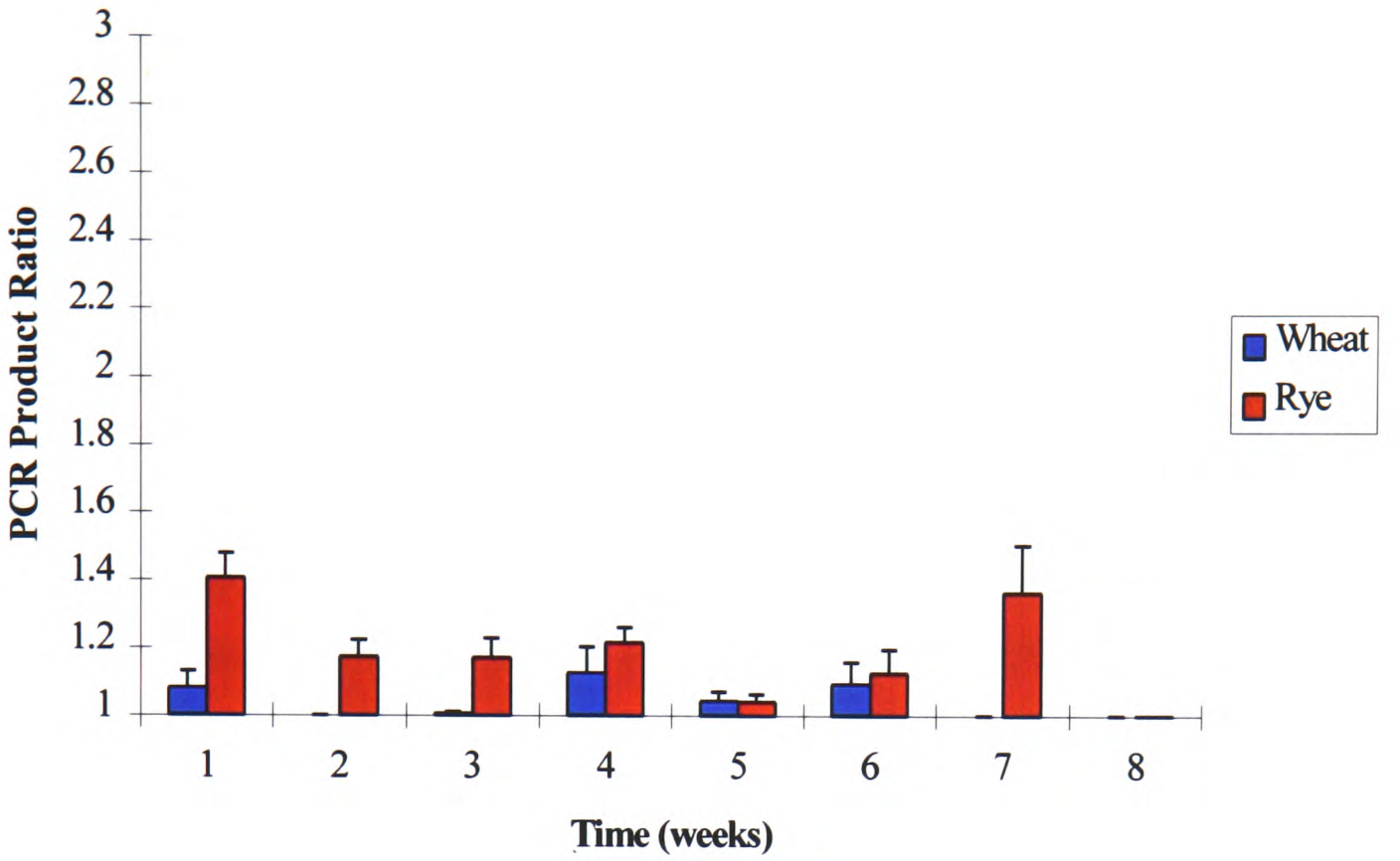


Figure 5.16: Graphs showing the mean amount of fungal DNA expressed as a PCR ratio in wheat and rye plants inoculated with W x R hybrid C41 and inter-specific hybrid DK over an infection period of 8 weeks. Error bars represent the standard error for each mean.

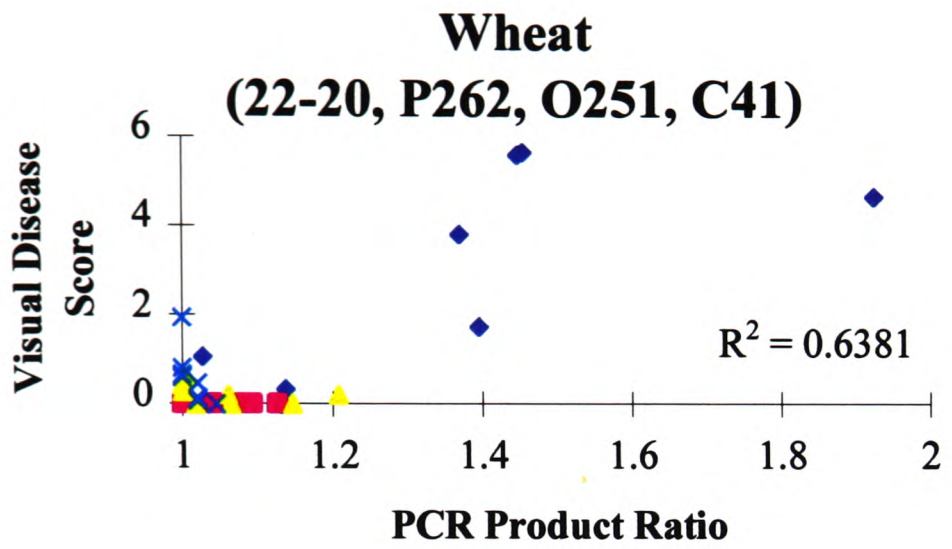
The amount of DNA from wheat and rye hosts inoculated with the W x R hybrids P262, O251, C41 was approximately 2/3 less on wheat and a 1/3 less on rye compared to the amount of DNA found on wheat and rye hosts inoculated with strain 22-20. The amount of DNA found on wheat and rye hosts inoculated with the inter-specific hybrid DK was only slightly less than that found on wheat and rye hosts when inoculated with strain 22-8.

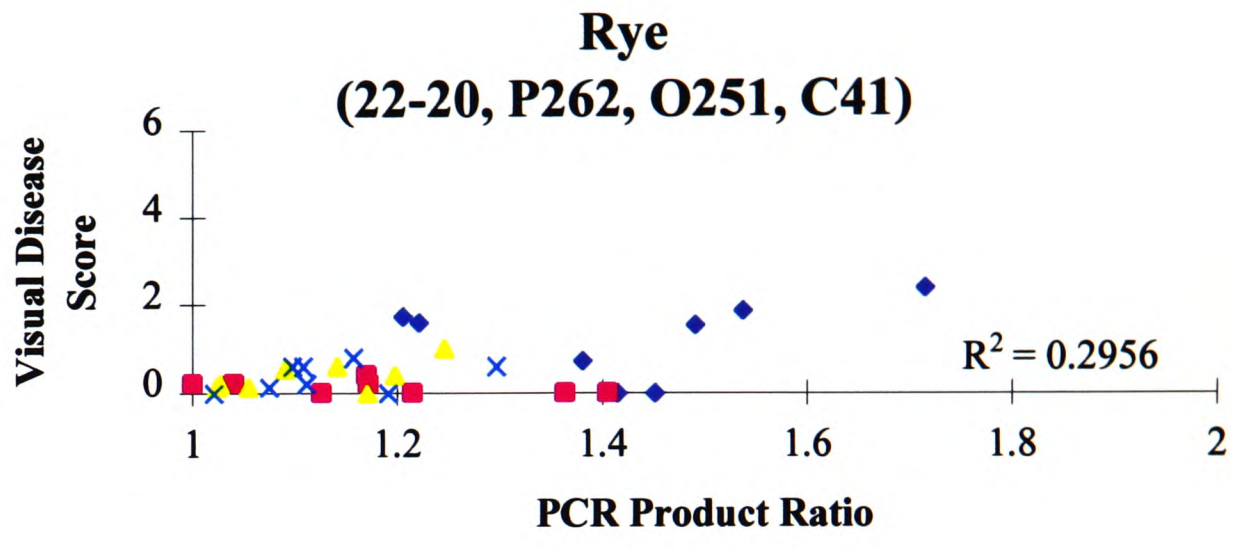
It is not possible to directly compare the PCR ratios obtained by the R and W primers and competitor templates because the competitors are different and thus an estimation is always made of their concentrations. Previous studies using standard curves of known quantities of fungal DNA have showed that a lower PCR ratio from an R-type is required to give a similar quantity of DNA from a W-type (Nicholson *et al.,* 1997). This investigation used a lower quantity of DNA in the reaction mixture and different amounts of competitor templates from the study which produced the standard curves. To compare the W and R primers directly new standard curves would need to have been made. However there was insufficient time to do this in this work. After taking the above information into account, it could be suggested that the amount of fungal DNA from both hosts infected with the R-type strain 22-8 was generally greater than with the W-type strain 22-20.

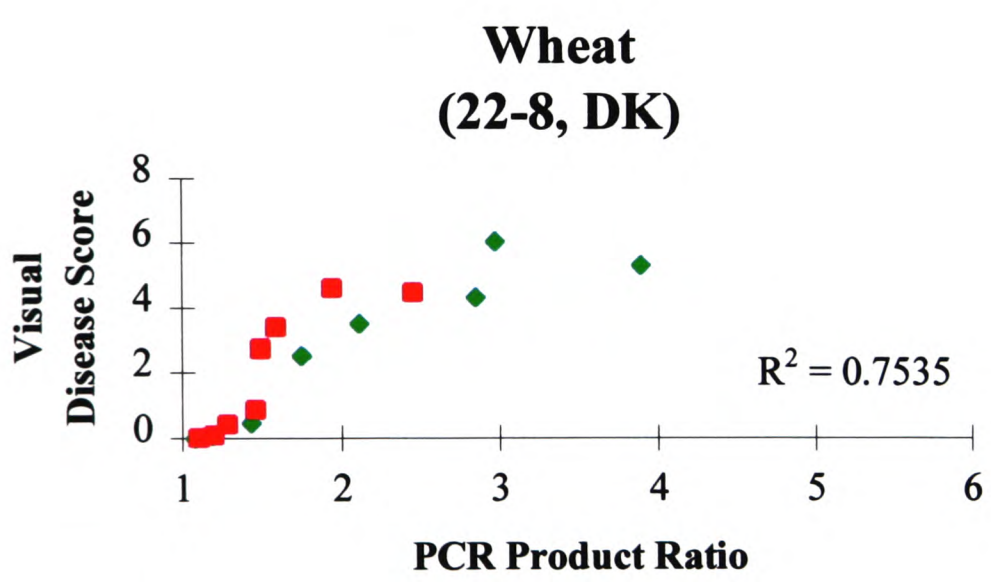
The fungal DNA content as estimated by the PCR product ratios of the wheat and rye plants was compared to the mean disease score of these plants. Figure 5.17 shows these relationships between wheat and rye with the data being combined for the W-type strain 22-20 and hybrids P262, O251 and C41 and combined for the R-type strain 22-8 and hybrid DK. By fitting a linear relationship to the results it indicates significant ($P<0.05$) positive correlations between the amount of estimated DNA and the disease infection score for wheat and rye respectively. Thus the higher the amount of estimated DNA the greater the penetration of the disease symptoms. It can be seen however that a linear relationship is not the best representation of the data and a more complex relationship is occurring ($R^2=0.6381$ on wheat and $R^2=0.7535$ on rye using

strain 22-20 and hybrids P262, O251 and C41. R^2=0.7168 on wheat and R^2=0.7056 on rye using strain 22-8 and DK).

Figure 5.18 compares the fungal DNA content as estimated by the PCR product ratios of the wheat and rye plants to the mean leaf sheath to which infection plaques were found. By fitting a linear relationship to these results significant (P<0.05) positive correlations were found between the mean amount of fungal DNA and the mean leaf sheath to which infection plaques were found. A linear relationship is however a poor representation of the data and a more complex relationship is occurring (R^2=0.1989 on wheat and R^2=0.1717 on rye with strain 22-20 and hybrids P262, O251 and C41 combined and R^2=0.6085 on wheat R^2=0.3861 on rye with strain 22-8 and hybrid DK).







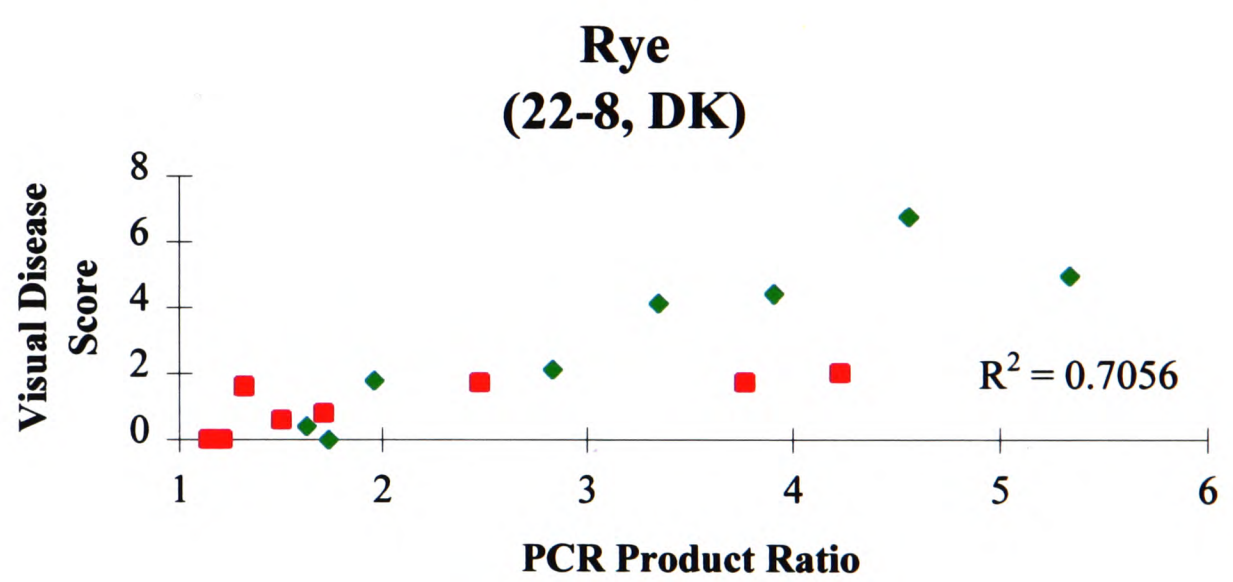
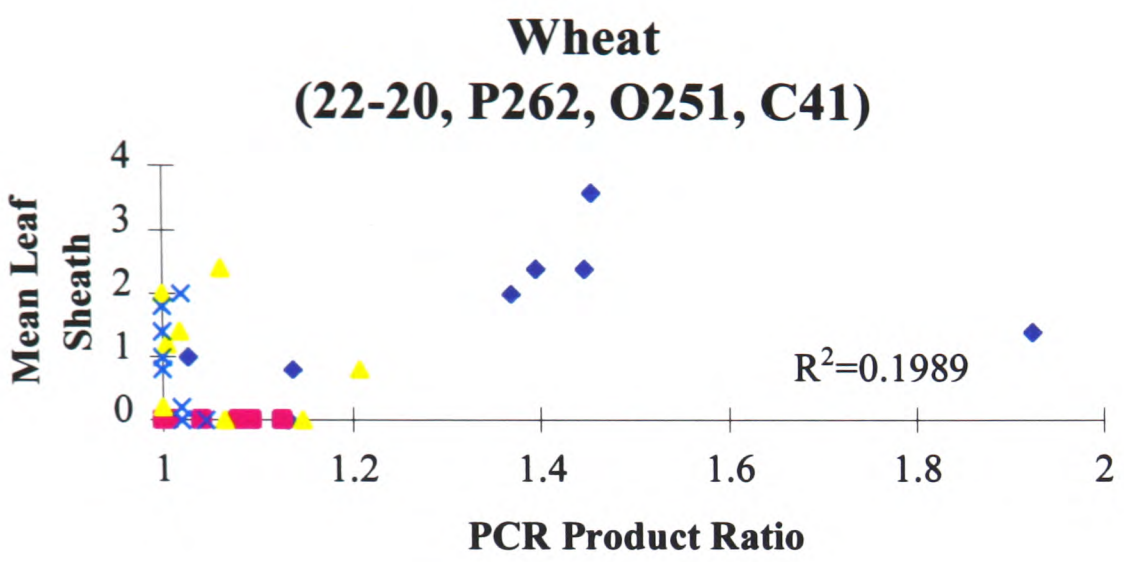
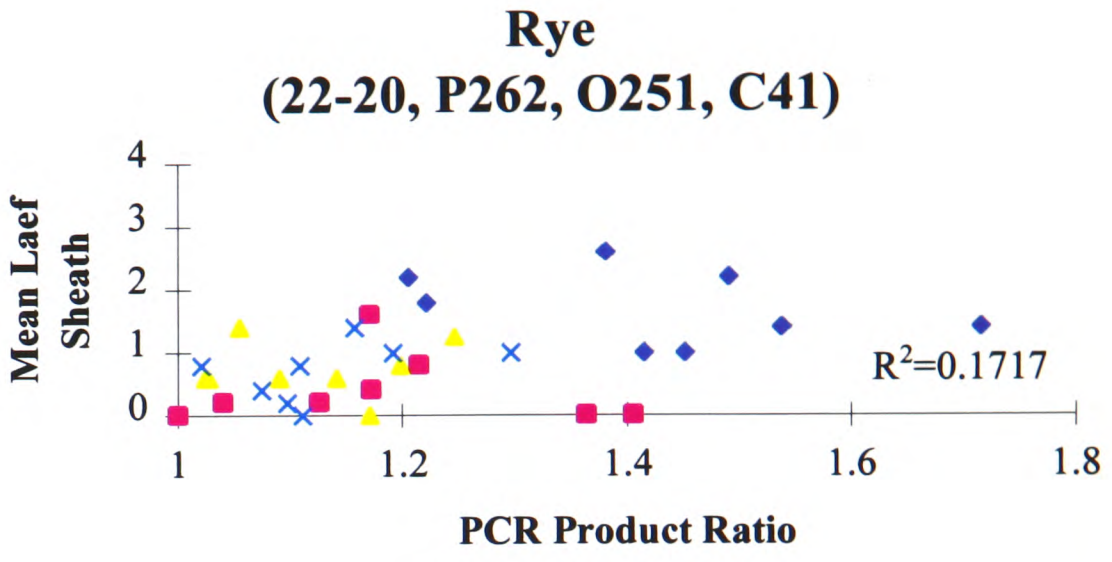
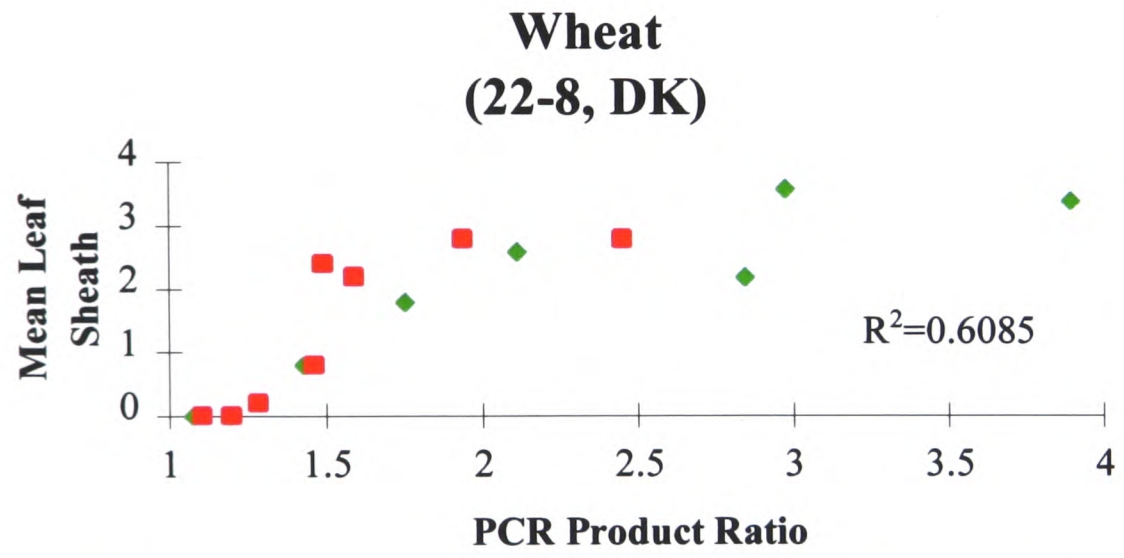


Figure 5.17: Graphs showing the relationship between visual disease assessments (mean 15 plants) and an estimation of fungal DNA (mean 10 plants) of wheat and rye plants inoculated with parental strains 22-20 ♦ and 22-8 ♦, and hybrids C41 ■, P262 ▲, O251 × and DK ■.







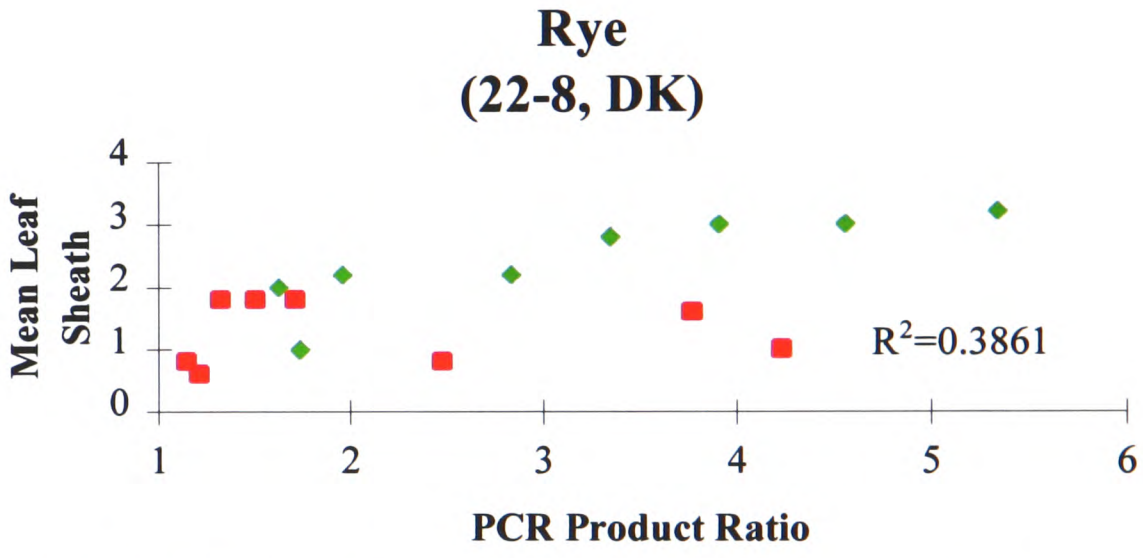


Figure 5.18: Graphs showing the relationship between the leaf sheath to which infection plaques were seen (mean 5 plants) and an estimation of fungal DNA (mean 10 plants) of wheat and rye plants inoculated with parental strains 22-20 ♦ and 22-8 ♦, and hybrids C41 ■, P262 ▲, O251 × and DK ■.

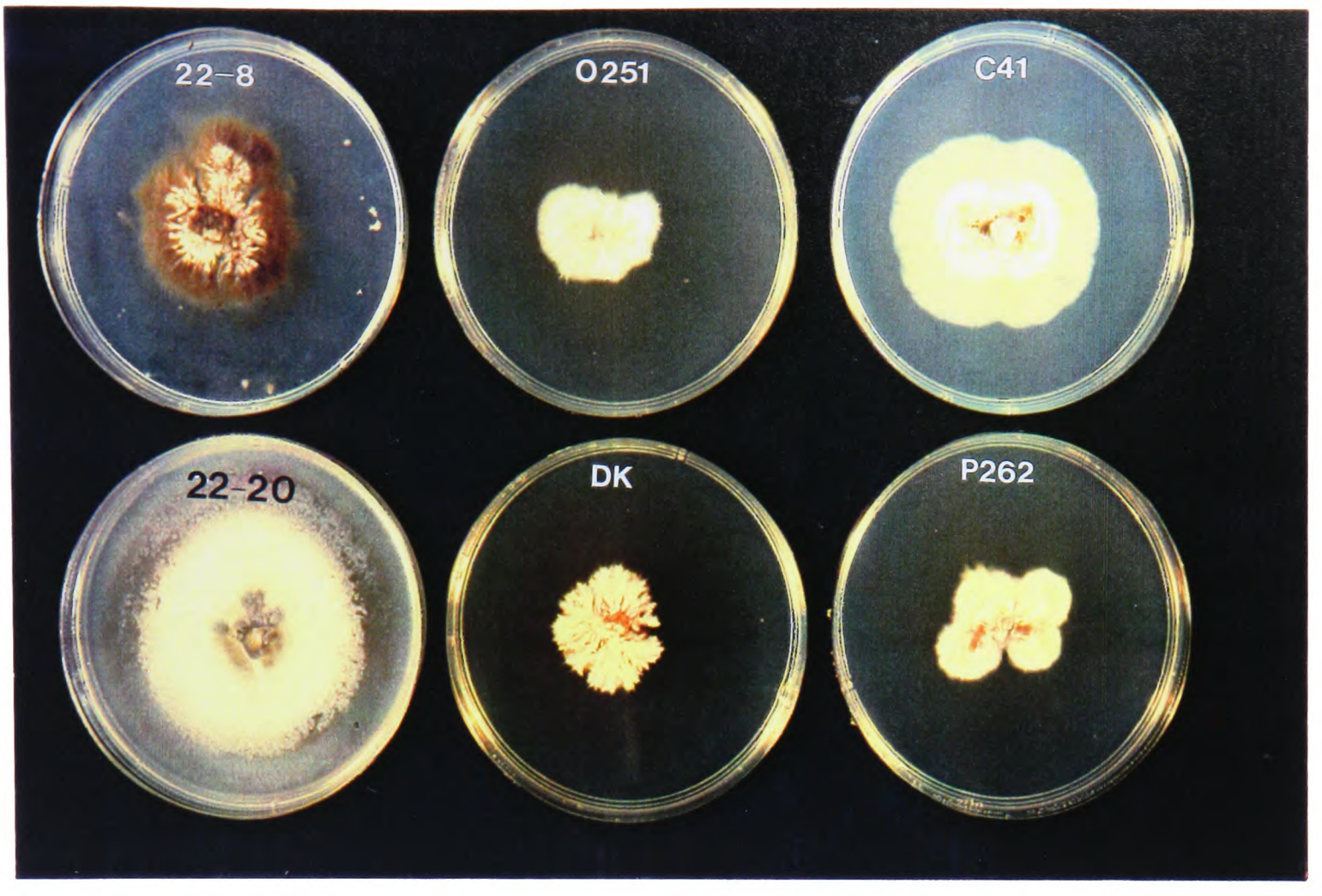
Plate 5.1

The parental strains, hybrids and method of inoculation of conidia on to wheat and rye seedlings.

A) The W and R-type parental strains 22-20 and 22-8, W x R hybrids P262, O251, C41 and inter-specific hybrid DK grown on MYG at 19°C in the dark.

B) Wheat seedlings showing inoculation of spore suspension using polyvinyl tubing and the layer of vermiculite present to maintain humidity required for infection.

A



B

Plate 5.2

Microscopic infection structures produced by *P. herpotrichoides* on wheat.

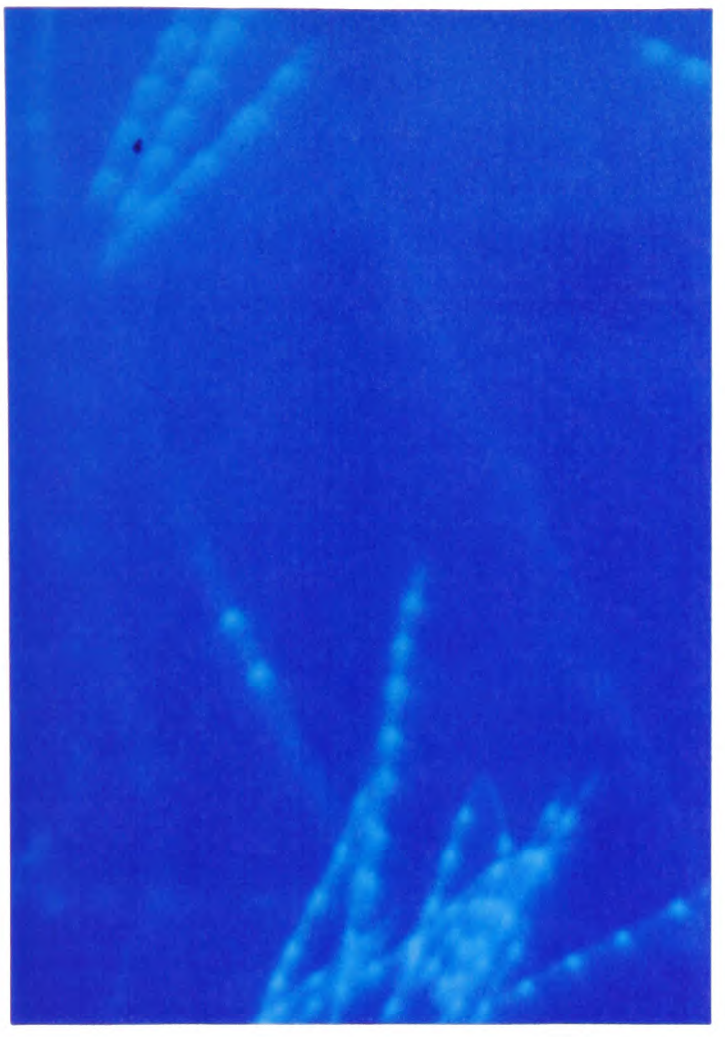
A) Conidia from W-type strain 22-20, magnification x 400.

B) W-type infection plaque produced by W-type strain 22-20, magnification x 400.

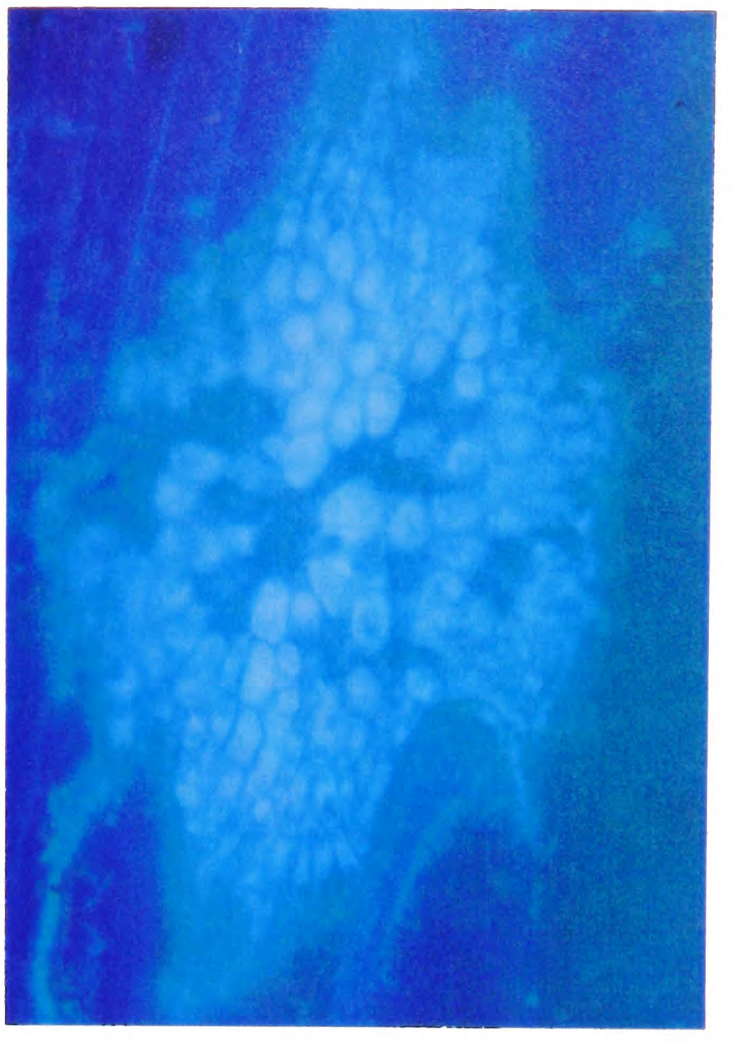
C) R-type infection plaque produced by R-type strain 22-8, magnification x 400.

D) Runner hyphae produced by W-type stain 22-20, magnification x 400.

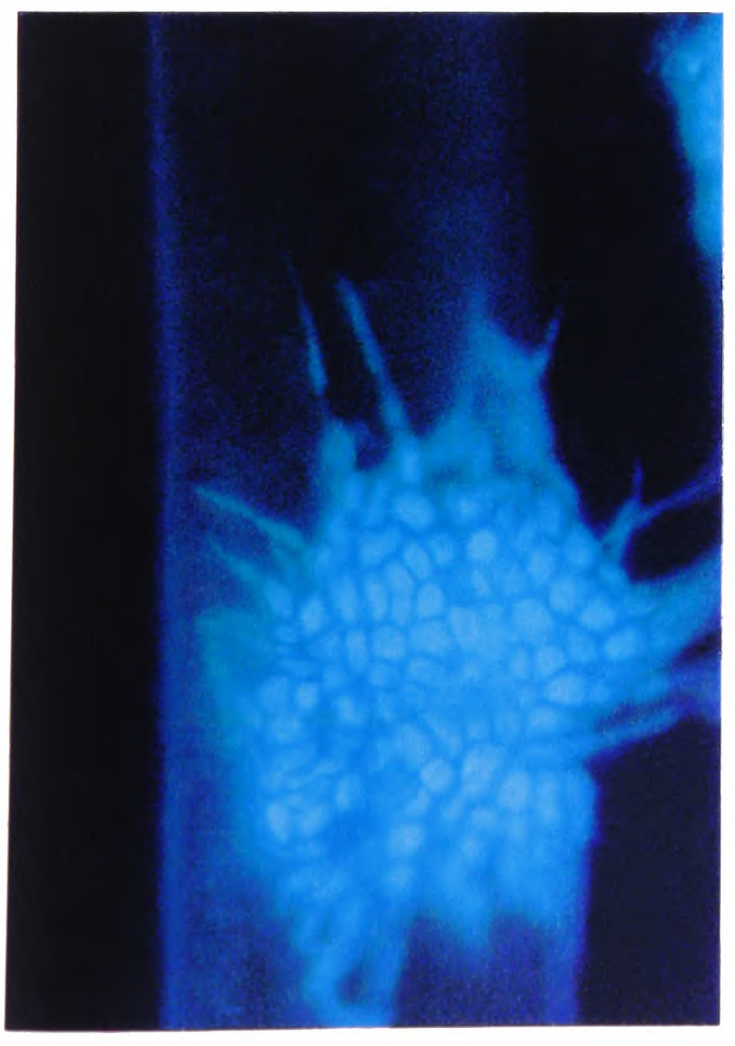
A



B



C



D

Plate 5.3

Microscopic infection structures produced by *P. herpotrichoides* on wheat.

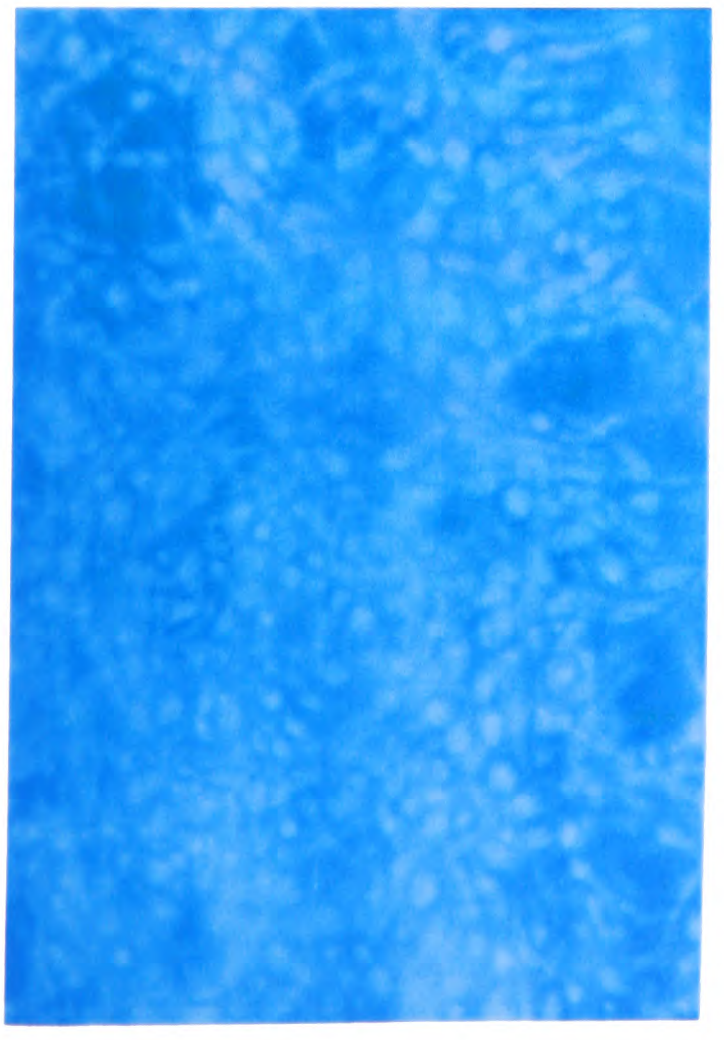
A) Mycelial plate composed of runner (r) and superficial hyphae (s) produced by strain 22-20, magnification x 400.

B) Aggregated W-type plaques produced by W-type strain 22-20, magnification x 400.

C) Immature infection plaque produced by the W x R hybrid P262, magnification x 400.

A

B

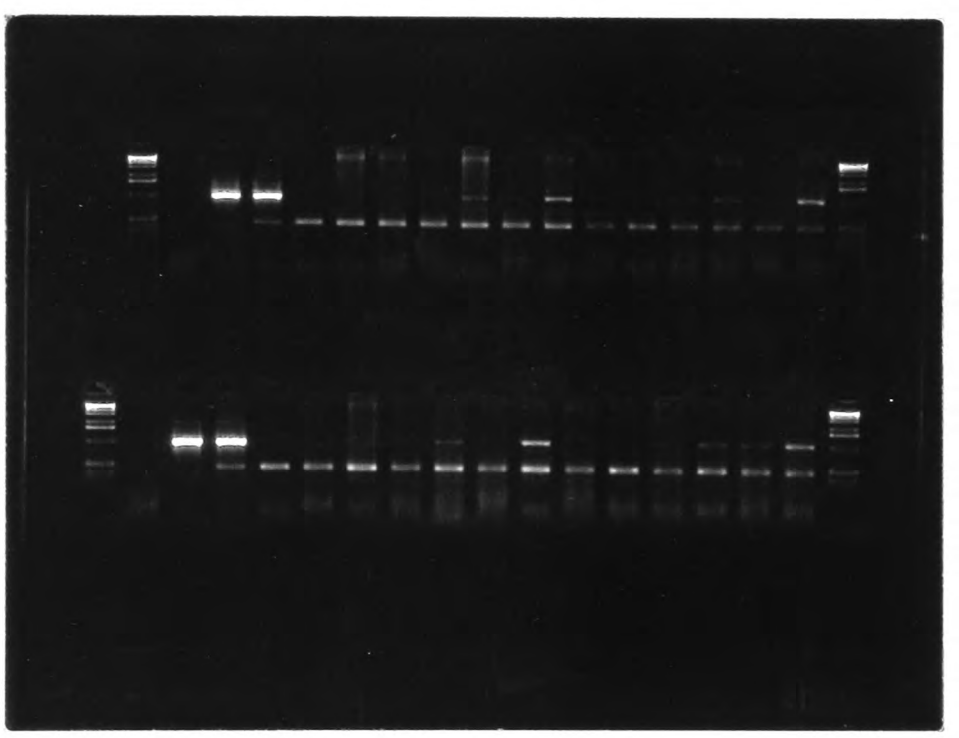


C

Plate 5.4

Competitive PCR of DNA from wheat samples inoculated with strain 22-20 and W x R hybrids P262, O251 and C41 amplified using the W primers TY16F/R in the presence of the W competitor template. The amplified fungal and competitor DNA products were then used to determine the PCR product ratios.

Top row: lanes 1 and 18 1.22kb ladder molecular weight marker, lane 2 negative control (no DNA template), lane 3 W-type template control, lane 4 W-type and W competitor template control, lane 5 W competitor template control, lanes 6-17 samples. Bottom row: lanes 1 and 19 1.22kb ladder molecular weight marker, lane 2 negative control (no DNA template), lane 3 W-type template control, lane 4 W-type and W competitor template control, lane 5 W competitor template control, lanes 6-18 samples.


Fungal (1.05kb)
Competitor (0.47kb)

5.4 Discussion

The assessment of disease symptoms over time indicates that initially the W-type strain was more pathogenic to wheat than the R-type strain. However, by the end of the infection period the depth of penetration of disease symptoms produced by both W and R-type strains was equal. The R-type strain was more pathogenic to rye than the W-type strain which was only slightly pathogenic on this host species. The R-type strain was approximately equal in its pathogenicity to wheat and rye. This suggests that host species adaptation has occurred for the W-type on wheat. These findings are similar to those of other pathogenicity experiments in glasshouses and controlled environment cabinets. Brown *et al,* (1984) found that the W and R-type strains were equally pathogenic to wheat, and the R-type strains have been found to be equally pathogenic to wheat and rye (Lange de la Camp, 1966; Scott *et al.,*1975; Hollins *et al.,* 1985). The R-type strains have also been found to be more pathogenic to rye than the W-type strains (Mauler and Fehrmann, 1987b). Other studies have found different pathogenicity results which contradict the present work and the above mentioned studies. For example the W-type strains have been found to be more pathogenic to wheat than the R- type strains (Fitt *et al.,* 1987; Higgins and Fitt, 1985b; Sanders *et al.,* 1986) and the R-type strains have been found to be more pathogenic to wheat than the W-type strains (Hollins *et al.,* 1985). Both the W and R-type strains have also been found to be more pathogenic to wheat than rye (Creighton *et al.,* 1989; Julian *et al.,* 1994). In the above mentioned studies describing the various pathogenicity results of the W and R-types on wheat and rye seedlings various inoculation techniques were used. The present work used a spore suspension of 2 x 10^5 spores ml^{-1} pipetted into polyvinyl tubing for inoculation of the coleoptile. In the studies by Fitt *et al,* (1987), Creighton *et al,* (1989) and Mauler and Fehrmann, (1987b) filter paper discs soaked in a spore suspension of 1 x 10^6 or 1 x 10^5 spore ml^{-1} were placed next to the coleoptile or between the coleoptile and the first leaf sheath. Mycelial collars made from agar and strains cultured on wheat straw have also been used as inoculum sources in the experiments of Fitt *et al,* (1987) and Scott *et al,* (1975) respectively. It appears that as long as infection can take place the method of

inoculation does not correlate with the pathogenicity results seen for the W and R-types. In other pathogenicity studies inoculated plants have developed lesions at a range of temperatures from 6-18°C, which implies that infection can take place over this range (Fitt *et al.,* 1988). The assessment of lesion development in these other studies has been assessed at a single timepoint, usually 6-12 weeks after inoculation. In the present study disease symptom development was monitored at weekly intervals giving a more accurate understanding of lesion development. The temperatures used in the present study (6°C night and 12°C day) were similar to those used by Scott *et al,* (1975) (average temperatures of 9, 7 and 11°C) who found similar pathogenicity results to those seen in the present work. Experiments by Fitt *et al,* (1987) and Creighton *et al,* (1989) used warmer temperatures (10°C night and 15°C day) and different pathogenicity results were seen. Temperature has been shown to influence the pathogenicity of *P. herpotrichoides* (Bateman *et al.,* 1990; Lange de la Camp, 1967). As already mentioned in the discussion of chapter 4 regarding wheat pathogenicity seedling tests, no differences were found between W and R-types at 10°C (Brown *et al.,* 1984) but R-types were more pathogenic than W-types at 7°C (Hollins *et al.,* 1985) and W-types more pathogenic than R-types at 18-22°C (Higgins and Fitt, 1985a; Sanders *et al.,* 1986). It has been shown that wheat and rye cultivars differ in their resistance to *P. herpotrichoides* (Scott *et al.,* 1976; Scott and Hollins, 1977; Mauler and Fehrmann, 1987a; Davies and Gareth Jones, 1970; Bateman *et al.,* 1985). Different cultivars of wheat and rye have also been used in the above mentioned pathogenicity experiments. Mauler and Fehrmann (1987b) used wheat cultivars Diplomat and Jubilar whereas Fitt *et al,* (1987) and Creighton *et al,* (1989) used the cultivar Armada. The work presented in the current study used the cultivar Beaver. Similarly for rye the current study used the cultivar Halo, whereas the study by Creighton *et al,* (1989) used the cultivar Dominant. Hence the direct comparison of pathogenicity experiments should done with caution especially when there is more than one variable between the experiments. Pathogenicity tests between wheat and rye are not reliable unless standard conditions and cultivars are used and a large number of strains must be tested to distinguish reliably between the two pathotypes.

Similar observations have been found for the W and R-type pathogenicity of adult wheat plants (Schreiber and Prillwitz, 1986; Higgins and Fitt, 1985b). Experiments have shown that disease symptoms on wheat appeared later when inoculated with the R-type strain suggesting differences in W and R-type epidemiology (Goulds and Fitt, 1990; Goulds, Bateman and Fitt, 1987; Cavelier *et al.,* 1987). Cool weather is thought to favour the development of R-type strain on wheat (Goulds and Fitt, 1990; Bateman, 1993). However the relationship between pathogenicity to seedlings (assessed on leaf sheaths) and pathogenicity to adult plants (assessed on stems) has been found to be poor (Higgins and Fitt, 1985b) with the ranking of isolates differing between field and glasshouse experiments (Scott *et al.,* 1975). The results obtained from pathogenicity experiments are from an interaction of the inoculation method, the hosts plant species, cultivar and age, the intrinsic aggressiveness of the strains and the environmental conditions used.

Problems have arisen in the field when assessing eyespot severity by disease symptoms. If the disease incidence increases and plants with slight lesions are included the mean severity score may decrease (Fitt and White, 1988). Hence when assessing disease severity it has been found to be important to incorporate both incidence and severity data from eyespot lesions on leaf sheaths. This is important in understanding how aggressive the strains are on a particular host which may be used to determine their pathotype as well as in control methods applied. A penetration index has been developed and simplified to the number of leaf sheaths penetrated per infected plant multiplied by the proportion of infected plants (Higgins and Fitt, 1984; Higgins, Fitt and White, 1986). ADAS advice in 1986 was that unless lesions have penetrated more than 2 leaf sheaths on more than 20% of shoots at growth stage 30 a spray decision should be delayed and further inspections made up to growth stage 32.

The microscopic infection structures produced by the W and R-type strains were the same on both wheat and rye. Similar infection patterns have also been found on different wheat cultivars. However the penetration of the coleoptile has been found to

be quicker on susceptible compared to resistant cultivars (Bateman and Taylor, 1976a; Scott, 1971). The infection structures seen in the current experiment were comparable to those originally identified by Daniels *et al,* (1991, 1995) on wheat seedlings. In the current study anticlinal alignment was found to occur only with the W-type strain on wheat. However on rye both W and R-types showed some anticlinal alignment. This may indicate different chemotrpoic/thigmotropic signals present in these hosts. Daniels *et al,* (1991) suggested, using a surface replica technique, that expansion of W-type hyphal cells in anticlinal cell wall grooves on wheat was not a simple topographical response. They also found that the W-types showed a pronounced intramural growth habit, predominantly in the middle lamella whereas R-types displayed a non-specific tissue invasion with very little intramural growth on wheat. Evidence of this was found on the wheat host in this experiment. Plates of mycelium developed on both hosts by both W and R-types; these consisted of superficial hyphae and runner hyphae which initiated colonisation on the leaf sheaths. Fixation and staining of the samples however may have removed other delicate or lightly attached structures.

Infection plaques were produced by the strains and hybrids. Daniels *et al.,* (1991) suggested that penetration into the host cell occurs via infection plaques cells. Due to a restriction of lateral expansion a small infection vesicle results at the cell tip, which along with an infection hyphae penetrate the host cell using both mechanical pressure and enzymatic degradation. In the current study a clear distinction was made between the infection patterns of the W and R-types on wheat and rye. On wheat and rye the W-type strain had a "slow steady" approach to infection plaque production whereas the R-type strain had a "fast and furious" approach which was immediate on rye but delayed by 2-3 weeks on wheat. A similar observation by Glynne (1952) led to the subsequent differentiation of W and R-types. There was also a much greater number of infection plaques produced by the R-type strain compared to the W-type. These differences between the W and R-types may involve a strategy of multiple penetration attempts by the R-type in the hope that some are successful whereas the larger

determinant, enabling the fungus to outgrow host defence responses (Daniels *et al.*, 1991). The structure of the host has been suggested to account for the difference observed in the penetration of eyespot in field inoculated wheat and barley where the rates of penetration of the leaf sheaths were the same. However because barley produces more leaf sheaths it takes longer to penetrate to the stem base compared to wheat (Goulds and Fitt, 1990). Structural differences can be observed between resistant and susceptible cultivars of wheat. Mauler and Fehrmann (1987a) observed that in resistant cultivars of wheat the time taken to penetrate the first leaf sheath was greater than susceptible cultivars, and penetration through successive leaf sheaths was slower in the resistant cultivar than in the susceptible cultivar. Studies on resistant wheat cultivars show distinct morphological differences compared to susceptible cultivars. A resistant cultivar "Roazon" had less mycelium than a susceptible "Etoile de Choisy", cultivar inoculated with *P. herpotrichoides* (Khan and Bouriquet, 1984; Khan, Bouriquet and Doussinault, 1986; Macer, 1966).

Infection plaques were produced after 2-3 weeks on wheat by the field strains and their formation can be correlated with the time cell-wall degrading enzymes were detected in the studies of Mbwaga *et al,* (1997). They detected high levels of enzyme activity 2 weeks after inoculation. Although they used a different cultivar of wheat and higher growth temperatures the presence of cell wall degrading enzymes at the time of infection plaque production supports the role of cell wall degrading enzymes in pathogenicity. Experiments investigating enzyme production in relation to infection plaque production on both wheat and rye over several weeks may also relate enzyme activity to disease symptom development.

It has been suggested that the growth of *P. herpotrichoides* in the plant is solely a function of temperature because there is always sufficient water at the site of the lesion to support growth (Ponchet, 1959). Ponchet (1959) further suggested that penetration of the leaf sheaths is a function of temperature where the number of leaf sheaths penetrated increased with increasing temperature over a range of 6-18°C.

However in the experiments of Higgins and Fitt (1985a) the rate of death of the leaf sheaths also increased with increasing temperature and increasing the temperature also increases competition from other microflora. Temperature has been shown to have little effect on differences between cultivars in the number of leaf sheaths penetrated (Scott, 1971). Higgins and Fitt, (1985a) suggested that wet cool weather (10°C night/15°C day) favoured the penetration of successive leaf sheaths and the establishment of stem lesions, although lesion development is not necessarily a function of temperature. It has been suggested that W and R-types may have different optimum conditions for infection (Fitt *et al.,* 1988). Further experiments altering the temperature may slow the establishment and rate of penetration of the W and R-types in wheat and rye and may help in the understanding of leaf sheath penetration and lesion development over time.

Both W and R-types showed secondary sporulation on both hosts although at different times after inoculation. The disease cycle of eyespot has been classed as being of primary spread (Rowe and Powelson, 1973b) by which the term "Simple Interest" (Van der Plank, 1963) can be applied, this being a disease which has a fixed inoculum source and the increase in disease with time is arithmetic. In microscopic studies on wheat Daniels *et al,* (1991) saw spores forming at the tips of short lateral hyphal branches. They found though that these spores which fell on to exposed leaf surfaces underwent limited differentiation to form superficial hyphae and appressoria, but penetration was rarely achieved. Similar conclusions have also been drawn from studies by Bateman and Taylor (1976a) and Higgins (1984). The spores seen in this experiment may be involved in secondary spread to tillers or neighbouring plants or they may contribute to the disease cycle by remaining on the leaf sheaths as they decay. They then act as an inoculum source for the following season along with later produced conidia on the straw stubble. Conidia produced on straw stubble is considered the dominant mechanism for infection of new shoots (Rapidly *et al.,* 1979).

Comparative studies between microscopic infection structures and disease symptoms indicate that infection structures of both W and R-types on wheat and rye precede disease symptoms. Fitt *et al,* (1988) and Daniels *et al.,* (1995) observed that by the time lesions were visible on the first leaf sheath, mycelium and infection plaque development and penetration of the second leaf sheath had occurred. This is thought to reflect the rate of leaf sheath penetration and host cell defence response. Lesions may fail to develop if the rate of outer leaf sheath death is greater than the rate of penetration of leaf sheaths, so that the outer leaf sheaths die and decay before the fungus has colonised the leaf sheaths beneath them (Fitt *et al.,* 1988).

The W x R hybrids produced few disease symptoms on wheat or rye; microscopically they produced W-type plaques on both hosts. However there was little penetration and leaf sheath colonisation. The infection plaques may have been unable to penetrate the leaf sheaths and thus gain the nutrients to allow colonisation. Studies have shown that osmophilic material, lignified papillae, stain halos and a hypersensitive reaction formed in epidermal cells in response to penetration in all cultivars of winter wheat. More papillae are formed in the first leaf sheath of resistant cultivars with fewer successful penetration sites occurring in them than in the susceptible cultivars at sites where papillae are present. Lignin is also found to be present in the papillae and halos surrounding penetration sites in all cultivars; the hypersensitive reaction is seen to have occurred only at sites with papillae. Highly pathogenic strains which rapidly penetrate leaf sheaths to establish visible lesions leave the host cell walls unchanged (Murray and Ye, 1986; Daniels *et al.,* 1991). Reduced infection plaque development and subsequent penetration may mean that the pathogen is unable to out pace the host cell response. Examination of the hosts for papillae would indicate that these are weakly pathogenic strains and help characterise the wheat and rye defence responses when inoculated with *P. herpotrichoides*.

The inter-specific hybrid produced typical R-type infection plaques which were probably inherited from the R-type parent although no microscopic studies have been

made on infection plaque structure produced by *P. anguioides,* the other parent of this hybrid. Infection structures were produced with leaf sheath colonisation and penetration being slightly less than the R-type parent ability to penetrate and colonise wheat and rye.

The W x R and inter- specific hybrids may have been affected by mutations caused by the UV mutagenesis used to generate selectable mutants which allowed manipulation of the parasexual cycle to produce the recombinants. These additional mutations may have not been visually apparent in *in vitro* growth studies but could have resulted in a lack of mucilage production thus giving poor spore adhesion to the leaf surface or a lack of adhesion to the runner hyphae which may be further investigated. Both factors aid colonisation of the leaf sheath and may prevent desiccation of the hyphae (Daniels *et al.,* 1991). Another factor involved in pathogenicity which may have been disrupted in these hybrids could be the production or secretion of cell wall degrading enzymes which are known to be secreted by *P. herpotrichoides* (Cooper *et al.,* 1988; Hanssler *et al.,* 1971). In wheat it has been seen that the preferential location of infection plaques produced by W-types is on cells overlying vascular traces and that the initial establishment of the pathogen is into vascular tissue (Daniels *et al.,* 1991). The stimulus for infection plaque formation from runner hyphae is unclear although near or complete exhaustion of nutrient in *in vitro* studies causes the formation of these plaques (Deacon, 1973). Infection structures recorded in *Rhizoctonia* are formed in response to host signals, possibly plant exudates for the production of mucilage necessary to promote close adhesion to the plant surface (Armentrout *et al.,* 1987). The receptors for possible host signals for infection plaque production may not be present or non functional in these hybrids thus reducing their ability to colonise the hosts.

Quantification of fungal DNA using competitive PCR supports the suggestion that the W and R-types have different infection strategies. The amount of fungal DNA did not increase with the W-type strain but it did increase with the R-type strain over the

assessment period. The "slow and steady" approach appears constant as the W-type strain proceeds through the leaf sheaths but the R-type, once successful in penetration, increases in amount as a greater inoculum potential is present once nutrients have been gained. It is difficult to directly relate microscopic disease infection structures to the amount of fungal DNA because the size of infection plaques was not precisely monitored. Thus the DNA from several R-type plaques may equal the DNA from a single W-type plaque. The relationship between successful penetration and plaque size would be a useful future investigation or using an alternative estimating fungal biomass perhaps by ergosterol content. Determination of the precise amount of fungal DNA would allow for direct comparison between the amounts of fungal DNA in both hosts by the W and R-strains. This could be achieved by producing standard curves with known quantities of DNA and the same amount of fungal competitor as was used in this experiment. The curves were not produced in the current work due to a limitation of time and resources. Equal quantities of the W-type strain were found in both wheat and rye plants. However the R-type strain produced more DNA on rye than wheat. These results contradict those found by Nicholson *et al,* (1997) who found when an isolated quantitative assessment was made of fungal DNA, the W-type strains colonised wheat more than rye. In addition they found that the amount of R-type strains was greater in all wheat and rye hosts compared to those inoculated with W-types. In field studies using wheat they found more R-type compared to W-type. However the amount of fungal DNA was less in field inoculated plots than comparative glasshouse tests. These differences may be explained by different conditions (glasshouse and field) used in these experiments and different age of glasshouse (12 weeks old) and field (growth stage 23) inoculated plants harvested.

Quantification of W and R-types using competitive PCR found positive correlations between disease symptoms and the amount of fungal DNA although the relationship appears not to be linear and varied for both W and R-types on wheat and rye. Other studies quantifying W and R-types using competitive PCR found also only a

moderate (R^2=0.6118) correlation between DNA and disease score for wheat and rye plants inoculated with W-types and a low correlation (R^2=0.3828) between the disease score and DNA when the plants were inoculated with R-types. In addition to this, no correlation has been found between disease score and the amount of DNA from field grown wheat seedlings for either pathotype (Nicholson *et al.*, 1997). These results indicate that the quantity of fungal DNA isolated from wheat and rye seedlings with similar disease scores might differ greatly depending on the pathotype involved.

The increase in DNA with the R-type in wheat and rye may reflect increasing colonisation. This may be advantageous for secondary spread of the fungus and increasing the inoculum source for the next year. Monitoring individual seedlings using competitive PCR would give a more accurate assessment of the development of disease in both wheat and rye.

Quantification of the fungal DNA from host plants infected with the W x R hybrids showed that there was more fungal DNA present in rye than in wheat. The level of infection in rye was similar to that seen by the W-type parental strain 22-20. A reduced amount of colonisation in wheat was unusual because *P. herpotrichoides* is a haploid organism (Davis and Jones, 1970) and it would be expected that as both parental strains could colonise wheat the W x R hybrids will colonise it at least as well as the worst parent. Additionally they should all be able to colonise rye at least as well as the W-type parent. The inter-specific hybrid should be able to colonise wheat and rye to the same level as its parents. A separate study of the ability of *P. anguioides* to colonise wheat and rye would need to be made to investigate this. These differences in the hybrid ability to infect wheat and rye compared to their parents could probably be attributed to physiological changes resulting from recombinant genomes.

These results using competitive PCR are different from alternative methods developed for quantifying the amount of *P. herpotrichoides* in infected plant tissue.

Polyclonal and monoclonal antibodies have been developed for use in enzyme linked immunosorbent assays (ELISA), which are also able to detect the pathogen in pre-symptomatic seedlings and naturally infected wheat. However, they are unable to differentiate W and R-types and in some cases cross react with *P. anguioides* and *P. aestiva* Nirenberg (Priestly and Dewey, 1993; Poupard *et al.,* 1994; Lind, 1990; Unger and Wolf, 1988). ELISA measurements have showed quantitative differences between resistant and susceptible wheat cultivars especially at later growth stages, with a correlation being found between visual eyespot scoring and ELISA results (Lind, 1992). An alternative method used a W-type GUS (β-glucuronidase) strain inoculated on to wheat seedlings. Here also a positive correlation was found between visual disease score ratings and GUS activity (de la Pena and Murray, 1994; Figliuolo *et al.,* 1998). Other studies have found that in degraded tissue a specific protein identified as plastocyanin (Pc) cannot be detected whereas it is abundant in the green tissue showing no disease symptoms. The amount of Pc has been correlated with the degree of tissue degradation determined using an ELISA test developed for this protein (Coff *et al.,* 1998).

There have been varying results comparing the relationship between disease symptoms and quantification/PCR products in other fungal pathogens. Specific PCR primers developed to detect *R. cerealis* Van der Hoven found no relationship between visual disease assessment and the results of the PCR (Nicholson and Parry, 1996). In comparison, quantification of colonisation of *V. albo-atrum* in alfalfa and *V. dahliae* in sunflower using specific competitive PCR found differences in infection similar to those obtained using conventional cytological or maceration and plating techniques (Hu, Nazar and Robb, 1993). Quantification of *Leptosphaeria maculans* (Desm.) Ces. & de Not. using competitive PCR revealed that lesion size in rape cultivars was associated with the quantity of DNA. In that study a decrease in the quantity of DNA coincided with sporulation, rapid necrosis and the onset of leaf senescence. (Mahuku, Goodwin and Hall, 1995). An assay based on the competitive PCR quantified *Glomus mosseae* (T. H. Nicolson & Gerd) Gerd & Trappe an arbuscular mycorrhizal fungus,

within leek plant roots. The competitive PCR and traditional microscopy assessments gave similar results in the colonisation of the roots (Edwards, Fitter and Young, 1997).

The other techniques developed for the quantification of fungal infection such as tissue maceration are laborious and limited in their specificity. These other alternative methods also use radio labelling of amplification products removed from gels and liquid scintillation counting of ratios of target to competitor fragments (Hu *et al.*, 1993; Moukhamedov *et al.*, 1994). Competitive PCR by the method of Nicholson *et al.*, (1996) can quickly quantify specific pathogens and it permits the relative proportion of a pathogen in a single plant to be estimated, and using DNA standards, to be related to absolute amounts of fungal DNA in a given mass of plant material. As quantification is based upon the relative degree of amplification within each sample comparisons may be made both within and between PCR assays when maintaining precise amplification. This will allow for better comparisons to be made in future studies.

The three methods of disease symptom assessment, microscopic assessment and fungal DNA quantification that were used in the current study to assess the W and R-types and hybrids of *P. herpotrichoides* lead to a more informative picture of fungal colonisation and lesion development on wheat and rye seedlings over a period of 8 weeks from initial inoculation. The W and R-types clearly showed different infection strategies on the different hosts which could be related to the disease symptoms seen. Quantifying the amount of fungal DNA in the hosts also confirmed that the W and R-types have different infection strategies. However caution must be taken if trying to relate the amount of fungal DNA to disease symptoms seen where a direct correlation is not apparent from the current study. Further work will need to be done in relation to the pathotype, host and environment if it is to be used in any way to predict the severity of disease symptoms. Thus, future studies of these relationships will allow better understanding of the disease and applications could then be made to increasing

CHAPTER 6

IDENTIFICATION AND ROLE OF MELANIN

6.1 Introduction

Growth *in vitro* of both W and R-types strains of *P. herpotrichoides* can be characterised by the presence of darkly pigmented fungal hyphae. Infection plaques formed *in vitro* in agar culture consist of cells with thickened pigmented walls (Deacon, 1973). In addition to this, studies on the development of infection plaques from both W and R-type strains on wheat seedlings showed that visible brown pigmentation occurred when the hyphal cells inflated and cell walls thickened (Daniels *et al.,* 1991, 1995). These dark brown to black pigments are suggested to be melanin similar to that identified in other fungi (Bell and Wheeler, 1986). The melanin pigments seen in *P. herpotrichoides* may be essential for penetration of host tissue and/or associated with fungal survival allowing structures to be resistant to extreme environmental conditions. The use of colour mutants has determined the role, location and biosynthetic pathway of melanins in several pathogenic fungi including *M. grisea, C. lagenarium* and *V. dahliae.* This chapter describes the production and characterisation of colour mutants from W-type strains of *P. herpotrichoides.* The melanin biosynthetic pathway present in this fungus was determined using DHN and DOPA melanin-inhibiting compounds and the antibiotic cerulenin. Ultrastructural studies of the wild-types and albino mutants determine the appearance and location of melanin granules in the hyphae. Genetic analysis of the colour mutants was carried out by back crossing with the wild-type strains. In addition to this, the role of melanin in this fungus was investigated.

6.2 Materials and Methods

6.2.1 Mutant production

Mutants of *P. herpotrichoides* W-type strains 22-20 and C87/631/1 were obtained by UV mutagenesis (Hocart *et al.*, 1993b). 10 ml conidial suspension of 1 x 10^6 spores ml^{-1} were placed in 9cm glass Petri-dishes each containing a paper clip on magnetic stirrers. UV doses (wavelength 254nm) were applied at 12 J m^{-2} s^{-1} using a UVGL58 Transilluminator. Conidia were kept in the dark after irradiation and before being plated out on to MYG agar to prevent photo-repair to the thiamine dimer bonds. Spore viability was estimated by dilutions in SDW achieving approximately 25 viable spores per 9cm Petri-dish. Assessments were made after 14 days for abnormalities in colour. Initial studies determined the time course of kill of the conidia using strain 22-20. Previous work (Hocart, 1987) found maximum mutagenesis occurred when conidial survival was <10%. From this information all further irradiations were then carried for 26 seconds (312 J m^{-2}) to obtain a conidial survival of <10%. Mutants were confirmed by visual observations of subcultures after continual sub-culturing to check for any reversions. Colour was always assessed subjectively and compared with the colour of mutants from *C. lagenarium* and *V. dahliae* (Kubo *et al.*, 1982a; Kubo *et al.*, 1987; Bell *et al.*, 1976b).

6.2.2 Characterisation of mutants

To determine if the pigments could be photo-induced the mutants and wild-type parents were point inoculated on to 9cm MYG agar plates and incubated at 19°C in the dark and light. The effect of different growth media on hyphal colouration was assessed by point inoculation of 9cm Petri-dishes containing Potato Dextrose agar (g l^{-1}, 39) and MYG agar; incubation was in the dark at 19°C. Growth was measured after 14 days as the mean of 2 perpendicular measurements across the colony using 3 replicate plates, and analysed using a single factor ANOVA. Hyphal colour was assessed visually after 21 days. To determine if the mutants could sporulate, hyphal plugs (5mm^2) were inoculated on to TWA and incubated at 16°C under near UV light (wavelength 360nm) for 10 days. Conidia viability was then determined by plating

out a 1ml aliquot of 2×10^5 spores ml^{-1} in SDW on to MYG agar plates and incubated at 19°C for 24 hours. Five hundred conidia for each mutant were examined using a binocular microscope and determined viable if germ tubes were present from the ends of the conidium. Statistical analysis of conidial viability was done using a single factor ANOVA. The presence of extracellular pigments secreted from the hyphae was assessed by growth in MYG liquid culture for 14 days. Mycelium was removed from the flasks by vacuum filtration using Whatman filter paper (grade 181), the liquid phase being collected in 10ml universal tubes. The presence of pigments in the media was visually assessed and compared to un-inoculated control media. In all the above characterisation experiments 3 replicates were used except for conidial viability which was replicated 4 times (i.e. 4 x 500 spores).

6.2.3 Complementation assessment

Complementation of the colour mutants was assessed by point-inoculation on to 9cm MYG agar Petri-dishes in pairs and also in a grid pattern of 12 mutants 1 cm apart. The plates were incubated at 19°C for 90 days allowing the hyphal fronts to meet. Assessment for complementation in a colour change was due to hyphal anastomosis and indicated vegetative compatibility. All combinations of the colour mutants and wild types were tested in triplicate, the controls being the same wild-type strain or colour mutant inoculated next to itself.

From the characterisation studies it was found that several colour mutants obtained from the wild-type strain C87/631/1 excreted pigments into the media. To examine the effect that these pigments had on the colour of the other mutants, they were inoculated into the centre of a 9cm MYG agar Petri-dish and also on to cellophane discs placed on top of the MYG agar (to allow for an assessment of colony colour when removed from the media). Six different colour mutants were then point inoculated in a circle around the pigment excreting strain (see Plate 6.2a). Plates were incubated at 19°C for 28 days allowing for pigments to be excreted into the media and for the colonies to grow. Assessments were then made on the mycelium colour.

The cellophane discs were removed from the agar before assessment to detect any hyphal colour change. This removed any false impression of colour change due to a change in the colour of the agar by the secreted pigments.

6.2.4 Melanin biosynthesis inhibiting compounds

Seven compounds were used to determine the biosynthetic pathway of the melanin associated with the pigmented hyphae of *P. herpotrichoides*. They were the antibiotic Cerulenin ([(2S)(3R)2,3-epoxy-4-oxo-7,10-dodecadienoylamide], from Sigma Co. Ltd.; the DHN-melanin inhibiting compounds (technical grade) pyroquilon (1,2,5,6-tetrahydro-4*H*-pyrrolo[3,2,1-*i,j*]-quinolin-4-one) (Novartis, Whittlesford, Cambridge), tricyclazole (5-methyl-1,2,4-triazolo-(3,4b)-benzothiazole) (DowElanco, Asia Pacific Sdn. Bhd, Malaysia) and fthalide (4,5,6,7-tetrachlorophthalide) (Kureha Chemical Industry Co., Iwaki, Japan); and the DOPA-melanin inhibiting compounds tropolone, diethyldithiocarbamic acid and 2-mercaptobenzothiazole, all from Sigma Chemical Co. Stock solutions of all DHN-melanin inhibiting compounds and 2-mercaptobenzothiazole were prepared by dissolving or suspending in 95% ethanol and then adding the appropriate amount of sterile de-ionised water. Cerulenin, tropolone and diethyldithiocarbamic acid were made using only sterile de-ionised water as they were insoluble in ethanol. All melanin inhibitors were added at 1, 10 and 100 μg ml^{-1} except for cerulenin which was added at 1 and 100 μg ml^{-1} to cooled MYG agar and the plates were then point inoculated with the wild-types and mutants listed in Table 6.7. Control plates contained 1μg ml^{-1} ethanol, 1μg ml^{-1} de-ionised water or were un-amended. Plates were incubated at 19°C in the dark. Mycelial growth (the mean of 2 perpendicular diameter measurements of the colony) and pigmentation colour were evaluated every 7 days for 28 days. The experiment had 4 replicates and the growth data was analysed using linear regression.

6.2.5 Examination for ultra-structural differences using transmission electron microscopy

Wild type strain 22~20 and its albino mutant MT183 were grown on cellophane discs placed on top of MYG agar for 21 days at 19°C in 5cm diameter plastic Petri-dishes. Approximately $1mm^3$ pieces of fungal material were fixed in 2.5% (v/v) glutaraldehyde and 2% paraformaldehyde in 0.025M sodium phosphate buffer pH7.2 for 2 hours. A 10% stock solution of paraformaldehyde was used consisting of 10g paraformaldehyde dissolved in 80ml water made up to 100ml. This milky solution was then heated to 60°C before the addition of drops of 1M NaOH until the solution became clear. Following fixation, the samples had 3 washes in 0.025M sodium phosphate buffer pH7.2 for 15 min each and were then post-fixed in 1% (w/v) osmium tetroxide in 0.025M sodium phosphate buffer pH7.2 for 2 hours. Following 3 further washes in 0.025M sodium phosphate buffer pH7.2 for 15 minutes each the samples were post-fixed in 2% uranyl acetate in water for 30 min. This was followed by 3 water washes for 15 minutes each. Dehydration was carried out with 10% increasing increments of ethanol until 90% for 15 minutes with the last 2 washes at 100% being for 30 minutes. The samples were then transferred to polypropylene oxide and embedded in Spurr's resin (Spurr, 1969). Polymerisation occurred at 70°C for 8 hours. Sections were cut using glassknives on an ultra microtome and collected on uncoated copper grids. Sections were stained for 5 minutes in 4% lead citrate (Reynolds, 1963). All steps were carried out at room temperature. Sections were examined using a Philips CM120 Biotwin transmission electron microscope using 60 kV accelerating voltage and electron micrographs taken.

6.2.6 Genetic analysis of the colour mutants

Sexual crosses were performed with the wild-type strain 22-20 and its colour mutants with the wild-type strain C87/631/1 and its colour mutants, wild-type strains C78/501, C91/761 and 22-433, all being of the opposite mating-type on straw agar and HP media in 5 cm Petri-dishes. Wild-type strain 22-20 had previously been designated mating type MAT1-1 (Dyer pers comm.). Strains C87/631/1, C78/501,

C91/761, 22-433 had been designated mating type MAT1-2 (Dyer, 1993; Nicholson *et al.*, 1995). Control plates contained only 1 strain or colour mutant. Crosses were set up as described by Dyer *et al* (1993b). Straw agar medium was composed of TWA and barley straw segments approximately 3cm long including a node. Straw segments were autoclaved twice for 30 minutes before addition to the TWA. HP medium was produced by soaking 20g of commercial mushroom compost in 1 L of water for 1 hour followed by boiling for 5 minutes. The mixture was filtered through 2 layers of muslin. Agar (15g) and malt extract (4g) were then added to the filtrate before it was autoclaved for 30 minutes. The solid bits from the mushroom compost were autoclaved twice for 30 minutes and placed on top of the cooled agar media. The media was inoculated singly or as a combination of strain and mutant by applying 30µl of a mixed spore suspension (1 x 10^6 spores ml^{-1}). Dishes were incubated in continuous white light at 10°C and assessed every 4 weeks for apothecial production. When mature apothecia formed a single apothecium was placed on petroleum jelly in the lid of a 5cm Petri dish over 3ml of SDW. Dishes were incubated for 24-48 hours at 15°C under white light. Samples (0.5ml) of SDW were then spread on to TWA in 9cm Petri-dishes. Dishes were left to dry and incubated at 15°C. Single ascospores were located by microscopy and transferred to MYG agar in 2 cm^2 compartments in a 10 cm^2 square Petri-dishes. These were then incubated at 19°C and a colour assessment made after 14 days.

6.2.7 Pathogenicity assessment of the colour mutants

An assessment was made on the pathogenicity of the wild-type strains and colour mutants listed in Table 6.1.

Wheat cv. Beaver seeds were sown in 6cm^2 plastic pots filled to within 2cm of the rim with peat based compost, 6 seeds per pot. Plants were maintained in plastic trays placed in Fitotron growth cabinets (16 hour photo-period, day temperature 12 ± 2 °C, night temperature 6 ± 2 °C, 80% relative humidity) and watered daily.

Fourteen day old seedlings were used for the inoculation. Three cm long pieces of polyvinyl tubing (internal diameter 4mm) were placed over them and pushed 1cm down into the soil, enclosing the seedling base. A 1.5ml aliquot of a 2 x 10^5 ml^{-1} spore suspension in SDW was pipetted into each tube and allowed to drain into the soil. Watering of the seedlings prior to inoculation improved the spore distribution over the coleoptile. After inoculation the pots were filled with vermiculite to maintain a high humidity around the stem base needed for infection (Plate 5.1b). After 72 hours the pieces of polyvinyl tubes were removed to prevent constriction of the stem base later on. Control plants were inoculated with SDW.

Three replicate pots were used for each strain/mutant. They were randomly allocated in a block design in the cabinet. The pots were watered by filling the propagation trays in which they stood with water as required to avoid overhead watering and thus prevent cross contamination. After 8 weeks, a visual assessment of pathogenicity was made using the infection scoring system described by Scott (1971) on the 3 pots (total 18 seedlings) as described in Section 4.2.12.

A microscopic assessment was made for the presence of infection plaques, hyphae and secondary sporulation with the fluorochrome ANS staining all surface structures. Nine stem bases (3 from each replicate pot) were examined by uprooting the seedlings and removing the coleoptile and subsequent leaf sheaths. These tissues were placed on a glass microscope slides and stained as described in Section 4.2.13. Observations were made for infection plaques on the coleoptile and each leaf sheath.

The pathogenicity data were analysed by the Kruskal Wallis analysis of variance by ranks (see 4.2.14) and differences between individual mutants and wild-types were identified using the Tukey Test for multiple comparisons of non-parametric data.

6.2.8 The effect of oxidation on the wild-types and colour mutants

The wild-type strains and colour mutants were point inoculated on to cellophane discs or on MYG agar in 5cm diameter Petri-dishes and incubated at 19°C for 21 days. The cellophane discs were removed and placed in the lids of the Petri-dishes to which 5ml of 1% sodium hypochlorite was added. To the colonies growing on MYG agar 5ml of 4% sodium hypochlorite was added. The time taken for the colonies to be bleached was measured. The experiment had 3 replicates. Data were analysed using a single factor ANOVA and the Tukey Test for multiple comparisons.

6.2.9 The effect of UV irradiation on the wild-types and colour mutants

The wild-type strains and colour mutants were point inoculated into 2 cm^2 compartments of 10 cm^2 square Petri-dishes. To standardise the depth of agar each compartment contained 1ml of MYG agar. The wild-types and mutants were incubated for 14 days at 19°C in the dark. Squares, 5x5mm were cut from the agar and placed in the lid of a 9cm plastic Petri dish. The agar squares were then irradiated using a UVGL58 Transilluminator at 12 J m^{-2} s^{-1}. An initial time course from 0 to 150 minutes irradiation using the buff and albino mutants strains MC87A and MC87AA determined the appropriate irradiation times in relation to mycelial survival. The wild-types and colour mutants were then irradiated for 0, 25, 50, 75 and 100 minutes. After irradiation the agar squares were kept in the dark before being transferred to fresh 10cm^2 Petri-dishes and incubated at 19°C in the dark. Mycelial re-growth from the agar squares was determined using a binocular microscope assessed on a daily basis. This was expressed as a percentage in comparison to the non-irradiated control agar squares. Three replicates were used for each irradiation time from 3 different compartments and the data were analysed using linear regression.

6.2.10 The effect of desiccation on the wild-types and colour mutants

Strains and colour mutants were point inoculated on to cellophane discs on MYG agar in 5cm diameter plastic Petri-dishes and incubated for 3 weeks at 19°C in the

dark. Pieces of cellophane on which hyphae was present were then cut using a scalpel to obtain an approximate fresh weight of 0.03g. The cellophane pieces were then placed in a desiccator containing a 2.5cm deep layer of silica gel. Phosphorus pentoxide was also tested as an alternative desiccant to silica gel with the rate of water loss being comparable. However the phosphoric acid released from the phosphorus pentoxide may have been toxic to the hyphal cells. Thus silica gel was chosen as the desiccant. An initial time course desiccating for 0 to 420 minutes using the wild-type strain C87/631/1 and green, buff and albino mutants MC87J, MC87A and MT178 respectively determined appropriate times of desiccation in relation to mycelial survival. All colour mutants and wild-types were then desiccated for periods of 120 and 360 minutes. The mycelial pieces were then removed and placed on MYG agar in 10cm square plastic Petri-dishes and incubated at 19°C in the dark. Mycelial re-growth from the cellophane pieces was determined using a binocular microscope assessed on a daily basis. This was expressed as a percentage in comparison to the non-desiccated control pieces. Three replicates, each from separate Petri-dishes, were used for each period of desiccation. The data were analysed using linear regression.

6.3 Results

6.3.1 UV Mutagenesis and colour mutant selection

Conidial survival from strain 22-20 in relation to UV dose is shown in Figure 6.1. It shows a slow decrease in conidial survival at lower UV doses (0-230 J m^{-2}) which is a characteristic of survival of multinucleate cells. UV doses of 230-480 J m^{-2} reduced conidial survival logarithmically proportional to the irradiation dose. Less than 1% survival was seen with UV doses greater than (420 J m^{-2}). Slight variation between conidial survival was seen in repeat experiments. However a similar pattern of survival was always observed. To produce the most mutants, a conidial viability of <10% was desired. Thus a dose of 312 J m^{-2} was used to irradiate all further conidia of the two darkly pigmented wild-type strains.

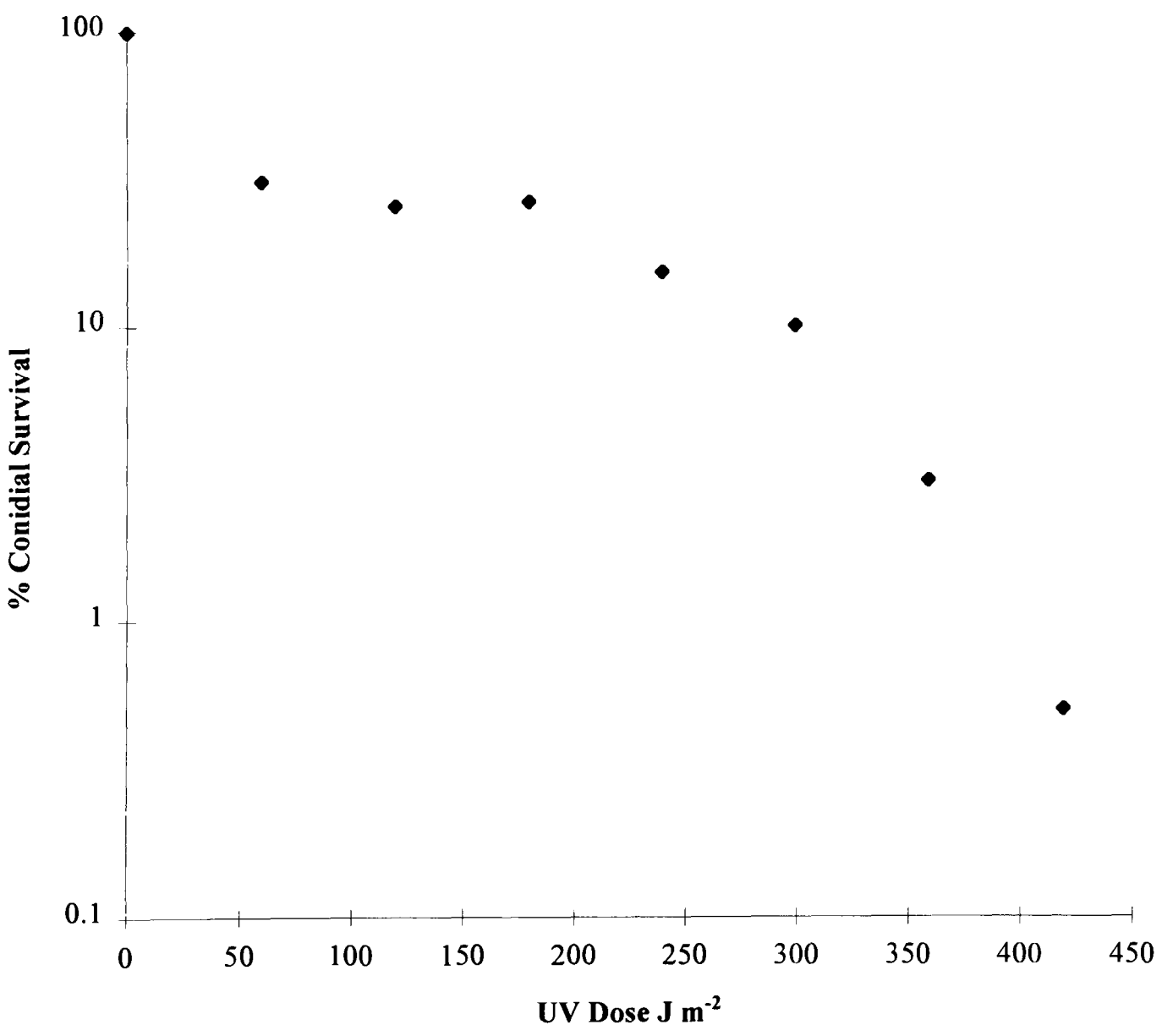


Figure 6.1: Graph showing the effect of increasing irradiation dose on conidial survival of wild-type strain 22-20.

Approximately 6000 conidia of each of the 2 darkly pigmented W-type strains, 22-20 and C87/631/1 were screened for colour abnormalities after UV irradiation. The colour mutants produced could be split into several phenotypic classes described as brown (Bro^-), green (Gre^-), buff (Buf^-), yellow (Yel^-) and albino (Alb^-) (mutation designation in brackets). From the wild-type strain 22-20 the albino mutants represented about 0.003-0.005%, yellow 0.004-0.006%, buff 0.0001%, green 0.0008-0.001% and brown 0.0008-0.001%. The frequency of the colour mutants from wild-type strain C87/631/1 was approximately double those from wild-type strain 22-20. Table 6.1 and Plate 6.1a and b show the colour mutants selected for study. They were selected on their colour intensity (light or dark), apparent normal growth, stable phenotype and ability to produce viable conidia on TWA. Generally, pigmentation was seen to occur in the mycelium of older colonies when hyphal growth was reaching the edge of the Petri dish, with the exception of the yellow pigment which was seen to occur concurrently with growth.

Table 6.1: Colour mutants selected for study produced by UV mutagenesis from the wild-types strains 22-20 and C87/631/1.

Wild-Type Parent	Mutant Code	Colour
22-20	MT215	Brown
22-20	MT156	Green
C87/631/1	MC87J	Green
22-20	MT38	Buff
C87/631/1	MC87A	Buff
C87/631/1	MC87C	Buff
C87/631/1	MC87E	Buff
C87/631/1	MC87K	Buff
C87/631/1	MC87D	Buff
22-20	MT88	Yellow
22-20	MT180	Yellow
C87/631/1	MC87CC	Yellow
22-20	MT178	Albino
22-20	MT183	Albino
22-20	MT77	Albino
22-20	MT141	Albino
22-20	MT485	Albino
C87/631/1	MC87AA	Albino
C87/631/1	MC87L	Albino
C87/631/1	MC87P	Albino

6.3.2 Characterisation of the colour mutants

A comparsion of the growth and pigmentation of the strains and colour mutants in the light and dark found that all of the albino mutants from wild-type strain 22-20 produced a pinky pigment in the light that was not seen when the mutants were grown in the dark. No other differences in pigmentation or its intensity were found between the light and dark. Table 6.2 shows the effect of light and different media on the pigmentation of the wild-types and colour mutants. When the wild-type and colour mutants were grown on potato dextrose agar a slight variation in pigment intensity was seen but no changes in colour. No significant differences were found between hyphal growth in the light or dark or between culture media. Significantly ($P<0.05$) slower growth rates were found with some of the colour mutants compared to the respective wild-type strain from which they were produced on the different media and conditions tested. It was observed that when colour mutants MT77 and MT141 were left for a long length of time (>120 days) slight grey pigmentation could be seen at the edge of the colonies. In these mutants, pigment production may have been slowed down and hence seen only occur when hyphal growth was restricted in the Petri-dish.

Table 6.2: The effect of light and media on the pigmentation of the wild-types and colour mutants.

Wild-type/Mutant	Pigment colour on MYG dark incubation	Pigment colour on MYG light incubation	Pigment colour on PDA
22-20	Black	Black	Black
MT215	Brown	Brown	Brown
MT156	Green	Green	Green
MT38	Buff	Buff	Buff
MT88	Yellow	Yellow	Yellow
MT180	Yellow	Yellow	Yellow
MT178	Albino	Pale Pink	Albino
MT183	Albino	Pale Pink	Albino
MT485	Albino	Pale Pink	Albino
MT77	Albino	Pale Pink	Albino
MT141	Albino	Pale Pink	Albino
C87/631/1	Black	Black	Black
MC87J	Green	Green	Green
MC87A	Buff	Buff	Buff
MC87D	Buff	Buff	Buff
MC87C	Buff	Buff	Buff
MC87E	Buff	Buff	Buff
MC87K	Buff	Buff	Buff
MC87CC	Yellow	Yellow	Yellow
MC87P	Albino	Albino	Albino
MC87L	Albino	Albino	Albino
MC87AA	Albino	Albino	Albino

No significant differences were seen in conidial germination between the wild-types and colour mutants with an average of 99% of conidia germinating. Hence no obvious phenotypic differences are present except for pigment colouration and growth rate.

Pigments were present in shake culture media from the wild-types and colour mutants and can be seen in Table 6.3 and Plate 6.2b. Wild-type strain 22-20 produced a pale green pigment into its media. However none of its colour mutants produced pigments into the media, with the media appearing just slightly cloudy from fungal growth. However wild-type strain C87/631/1 and its green and buff colour mutants

generally excreted the same colour of mycelium pigmentation into the media. The albino mutants from the same wild-type strain did not excrete any pigments into the media although again the media was slightly cloudy from fungal growth. This indicates that the same colour pigment in the hyphae is secreted into the media.

Table 6.3: Pigment excretion in to shake culture media by wild-type strains and their colour mutants.

Wild-type/ mutant	Colour on MYG agar	Pigment in media	Colour of pigment
Control	-	X	Clear
22-20	Black	X	Pale Green
MT215	Brown	X	Cloudy
MT156	Green	X	Cloudy
MT38	Buff	X	Cloudy
MT88	Yellow	X	Cloudy
MT180	Yellow	X	Cloudy
MT178	Albino	X	Cloudy
MT183	Albino	X	Cloudy
MT485	Albino	X	Cloudy
MT77	Albino	X	Cloudy
MT141	Albino	X	Cloudy
C87/631/1	Black	√	Black
MC87J	Green	√	Green
MC87A	Buff	√	Buff
MC87D	Buff	√	Buff
MC87C	Buff	√	Buff
MC87E	Buff	√	Buff
MC87K	Buff	√	Greenish
MC87CC	Yellow	√	Pale Buff
MC87P	Albino	√	Cloudy
MC87L	Albino	√	Cloudy
MC87AA	Albino	√	Cloudy

X - No pigments excreted into media
√ - Pigments excreted into media

6.3.3 Complementation assessment

Most combinations of mutants and wild-types failed to show any complementary colour changes. In several cases there was no contact between hyphal fronts. Where hyphal fronts met and no complementary colour changes were seen, it was taken to indicate vegetative incompatibility between the strains tested. In the cases where complementation occurred, darkly pigmented mycelium was at the interface of the colonies and can be seen in Plate 6.3a and b. Complementations were seen within mutants from the same parental wild-type and between mutants from the different parental wild-types (Tables 6.4, 6.5, 6.6). More complementations were seen between the wild-type strain 22-20 and its colour mutants, in particular the green mutant MT156 and yellow mutant MT88. The only complementation from wild-type strain C87/631/1 and its colour mutants was between the buff mutants MC87A and MC87C. Inter-complementation between strains and mutants occurred between the buff mutant MC87A and yellow mutant MT88.

Table 6.4: Complementation between pairs of wild-type strain 22-20 and its colour mutants.

Strain	Colour	22-20 Black	MT215 Brown	MT156 Green	MT38 Buff	MT88 Yellow	MT180 Yellow	MT178 Albino	MT183 Albino	MT485 Albino	MT77 Albino	MT141 Albino
22-20	Black	-	-	+	+	+	-	-	-	-	?	-
MT215	Brown	-	-	-	?	-	-	-	-	-	-	-
MT156	Green	+	-	-	+	+	-	-	+	-	+	-
MT38	Buff	+	?	+	-	+	?	?	-	-	-	-
MT88	Yellow	+	-	+	+	+	-	+	-	-	+	+
MT180	Yellow	-	-	-	?	-	-	-	-	-	-	-
MT178	Albino	-	-	-	?	+	-	-	-	-	-	-
MT183	Albino	-	-	+	-	-	-	-	-	-	-	-
MT485	Albino	-	-	-	-	-	-	-	-	-	-	-
MT77	Albino	?	-	+	-	+	-	-	-	-	-	-
MT141	Albino	-	-	-	-	+	-	-	-	-	-	-

Table 6.5: Complementation between pairs of wild-type strain C87/631/1 and its colour mutants.

Strain	Colour	C87/631/1 Black	MC87J Green	MC87A Buff	MC87D Buff	MC87C Buff	MC87E Buff	MC87K Buff	MC87CC Yellow	MC87P Albino	MC87L Albino	MC87AA Albino
C87/631/1	Black	-	-	-	?	-	-	-	?	-	-	-
MC87J	Green	-	-	-	?	-	-	-	?	-	-	-
MC87A	Buff	-	-	-	-	+	-	?	?	-	-	-
MC87D	Buff	?	?	-	-	-	-	?	?	?	-	-
MC87C	Buff	-	-	+	-	-	-	-	?	-	-	-
MC87E	Buff	-	-	-	-	-	-	-	-	-	-	-
MC87K	Buff	-	-	?	?	-	-	-	-	-	-	-
MC87CC	Yellow	?	?	?	?	?	-	-	?	?	?	?
MC87P	Albino	-	-	-	?	-	-	-	?	-	-	-
MC87L	Albino	-	-	-	?	-	-	-	?	-	?	-
MC87AA	Albino	-	-	-	-	-	-	-	?	-	-	-

\+ Complementation
\- No complementation
? No contact between hyphae of different strains

Table 6.6: Inter-complementation between pairs of wild-type strains C87/631/1 and 22-20 and their colour mutants.

Strain		Strain										
	Colour	22-20 Black	MT215 Brown	MT156 Green	MT38 Buff	MT88 Yellow	MT180 Yellow	MT178 Albino	MT183 Albino	MT485 Albino	MT77 Albino	MT141 Albino
C87/631/1	Black	-	-	-	-	-	-	-	-	-	-	-
MC87J	Green	-	-	-	-	-	-	-	-	-	-	-
MC87A	Buff	+	-	-	-	+	-	-	-	-	-	-
MC87D	Buff	?	-	-	-	-	-	-	-	-	-	-
MC87C	Buff	-	-	-	-	-	-	-	-	-	-	-
MC87E	Buff	-	-	-	-	-	-	-	-	-	-	-
MC87K	Buff	-	-	-	-	-	-	-	-	-	-	-
MC87CC	Yellow	?	?	?	-	?	?	?	-	?	?	?
MC87P	Albino	-	-	-	-	-	-	-	-	-	-	-
MC87L	Albino	-	-	-	-	-	-	-	-	-	-	-
MC87AA	Albino	-	-	-	-	-	-	-	-	-	-	-

\+ Complementation
\- No complementation
? No contact between hyphae of different strains

The buff colour mutants MC87A, MC87E, and MC87D from wild-type strain C87/631/1 were found to excrete a reddish pigment into the media. When the albino (MC87L, MC87AA, MC87P, MT178, MT485, MT187, MT141, MT77), yellow (MT88, MT180), and buff (MT38) colour mutants were grown on media containing this reddish pigment they gained the appearance of buff coloured mutants which can be seen in Plate 6.2a. To eliminate the possibility that pigments secreted into the media were giving the colonies a buff appearance the cellophane discs on which the colonies were growing were removed. The albino, yellow and pale buff colonies described above retained their buff appearance and when viewed with a binocular microscope the hyphal cells were clearly a reddish colour.

6.3.4 Melanin biosynthesis inhibitors

The DHN melanin-inhibiting compounds tricyclazole, fthalide and pyroquilon inhibited development of the black/dark brown melanin pigments associated with the wild-type W-type strains C87/631/1 ,22-20, C78/501 and the wild-type R-type strain 22-8. This was evident by the formation of mycelia with a reddish pigmentation instead of a dark brown to black pigmentation. A change in colour pigmentation was seen by the 21 day assessment of growth and colour (Table 6.7). The brown (MT215), green (MT156, MC87J) and buff (MC87K) colour mutants from the wild-type strains 22-20 and C87/631/1 also formed reddish mycelia in the presence of the DHN-melanin inhibiting compounds. The buff (MC87A), yellow (MT180), and albino (MT183, MC87AA) colour mutants showed no pigmentation colour change in the presence of these compounds (see Plates 6.4 a and b, 6.5 a and b). The optimum concentration at which colour inhibition occurred differed between compounds with an optimum concentration of 10 $\mu g\ ml^{-1}$ for the compounds fthalide and pyroquilon to 1 $\mu g\ ml^{-1}$ for the compound tricyclazole. At the highest concentration tested of 100 $\mu g\ ml^{-1}$ some of the strains and mutants were a cream colour but showed a buff colour at the optimum concentrations for fthalide, pyroquilon and tricyclazole pigmentation inhibition as described above.

Table 6.7: The effect of DHN-melanin inhibiting compounds (μg ml^{-1}) on mycelial pigmentation of *P. herpotrichoides* strains and their colour mutants grown on MYG agar.

Isolate	Description	Tricyclazole			Fthalide			Pyroquilon		
		1	10	100	1	10	100	1	10	100
22~8	Dark brown	++	+	C	+	++	C	+	++	+
C78	Dark brown	+	+	+	-	-	++	+	++	+
22~20 and its colour mutants										
22~20	Black	++	+	C	-	++	+	+	++	(+)
MT215	Brown	++	+	C	-	++	+	++	+	(+)
MT156	Green	++	+	C	-	+	+	+	(+)	C
MT180	Yellow	-	-	C	-	-	-	-	-	-
MT183	Albino	-	-	-	-	-	-	-	-	-
C87/63/1 and its colour mutants										
C87/631/1	Black	+	++	-	+	+	+	+	++	+
MC87J	Green	+	++	C	-	+	+	+	++	C
MC87A	Buff	-	-	-	-	-	-	-	-	-
MC87K	Buff	+	-	-	-	-	-	-	-	(+)
MC87AA	Albino	+	-	-	-	-	+	-	-	-

Colour changes compared to mycelia grown for 28 days on un-amended MYG agar

+	Colour change to pale buff	C	Colour change to cream
++	Colour change to buff	-	No colour change
(+)	Patchy colour change to buff		

The suggested sites of blockages in melanin biosynthesis for the colour mutants are seen in Fig 6.2. The albino and yellow mutants showed no colour change indicating that they contain a genetic block before the production of 1,3,6,8-THN (or 1,3,8-THN). The buff mutant also showed no colour change indicating a genetic block after the production of scytalone/vermelone. The colour of the buff mutants was similar to that obtained when tricyclazole, fthalide and pyroquilon are applied to the darkly pigmented wild-types 22-20, C87/631/1, C78/501 and 22-8, indicating that the block in these buff mutants is possibly due to the lack or non-function of the reductase enzyme which reduces 1,3,8-DHN to vermelone, this enzyme is the primary site of action of the DHN melanin-inhibiting compounds. The brown and green colour mutants showed colour changes indicating a genetic block after the production of vermelone The albino mutant MC87AA showed some reddish pigmentation in the centre of the colony at a concentration of 100µg ml^{-1} fthalide and 1 µg ml^{-1} tricyclazole. This mutant thus may not be pure albino.

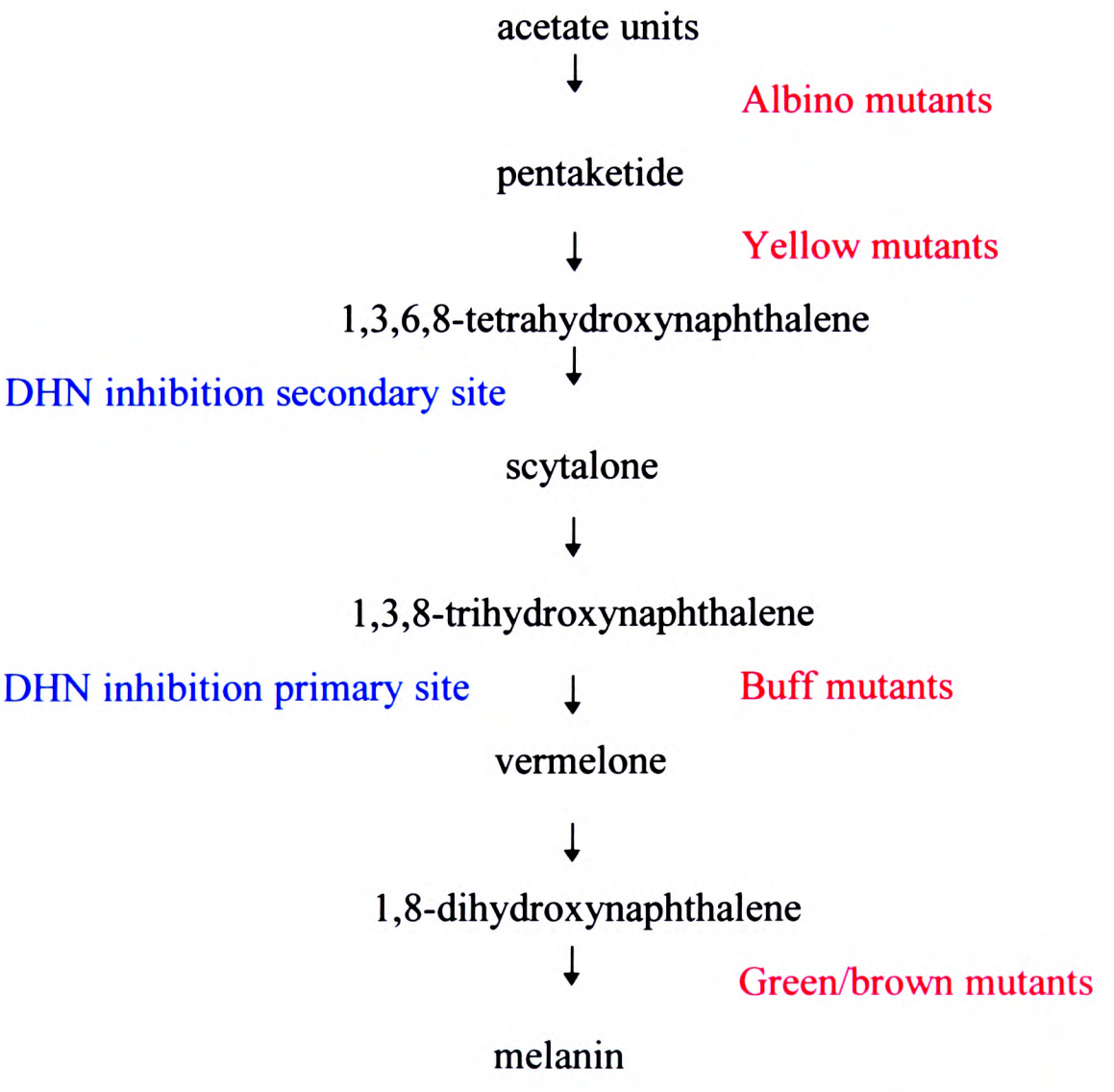
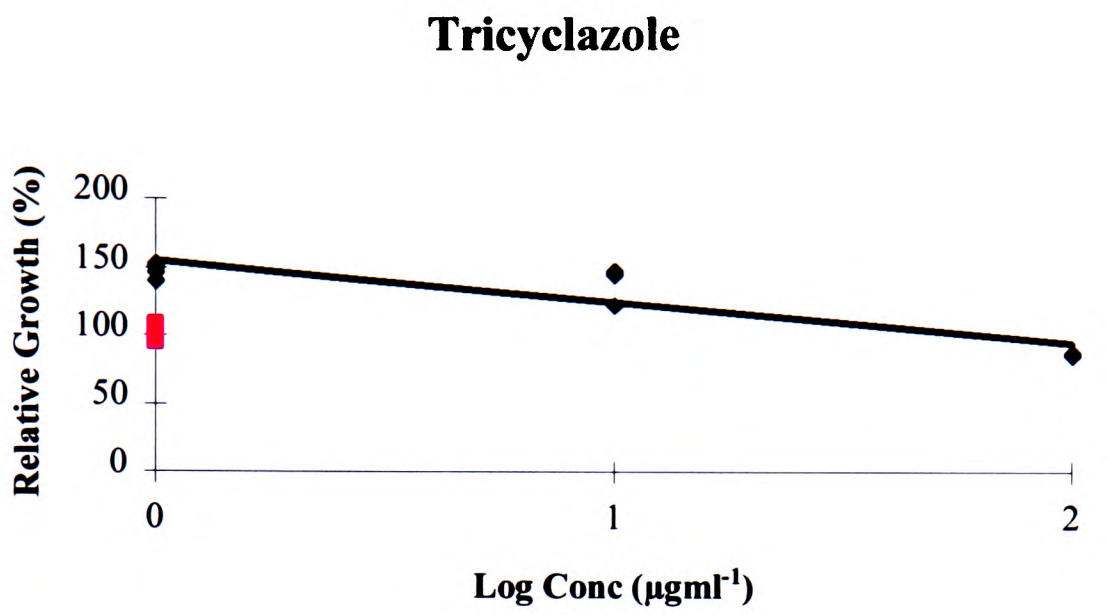
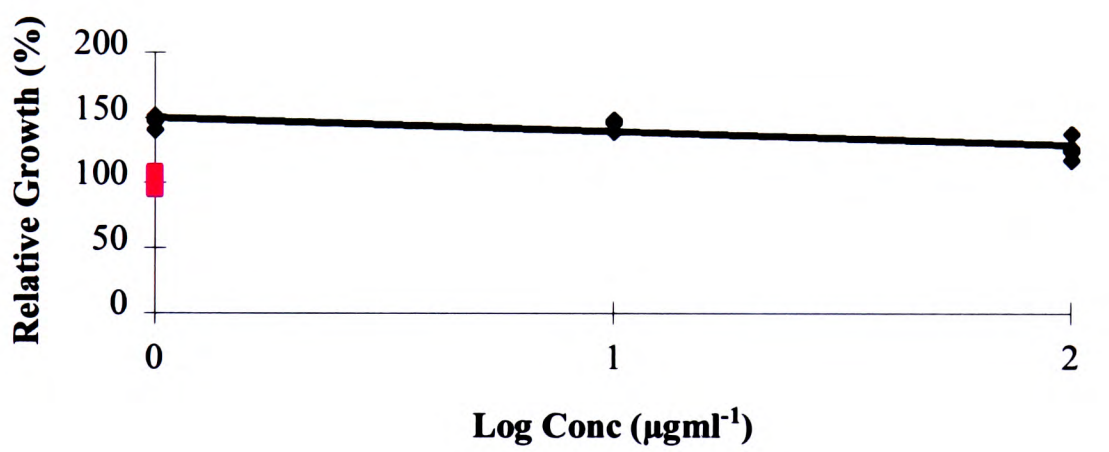


Fig 6.2: Possible sites of genetic blocks of the colour mutants.

The concentrations that interfered the most (i.e. the maximum reddish pigmentation seen) with the melanin biosynthetic pathway of the DHN-melanin inhibiting compounds, tricyclazole, fthalide and pyroquilon were found to be non-fungitoxic in that they did not inhibit vegetative growth. Growth was analysed using a linear regression analysis with a log transformation of the compound concentration. Figure 6.3 shows the effect of the different concentrations on the relative growth assessed after 21 days of the wild-type strain 22-20. It can be seen that initially at low concentrations all 3 compounds increased mycelial growth. This may be due to compounds causing stress to the fungus which may cause an increase in radial growth and/or less hyphal branching. Generally increasing the compound concentrations was found to linearly decrease vegetative growth. An example of this from wild-type strain 22-20 is where it was found that at 100μg ml^{-1} tricyclazole produces a relative growth of 93%, fthalide 129% and pyroquilon 117%. A similar pattern of an initial increase followed by a linear decrease with increasing compound dose on vegetative growth was obtained for all wild-types and colour mutants tested.





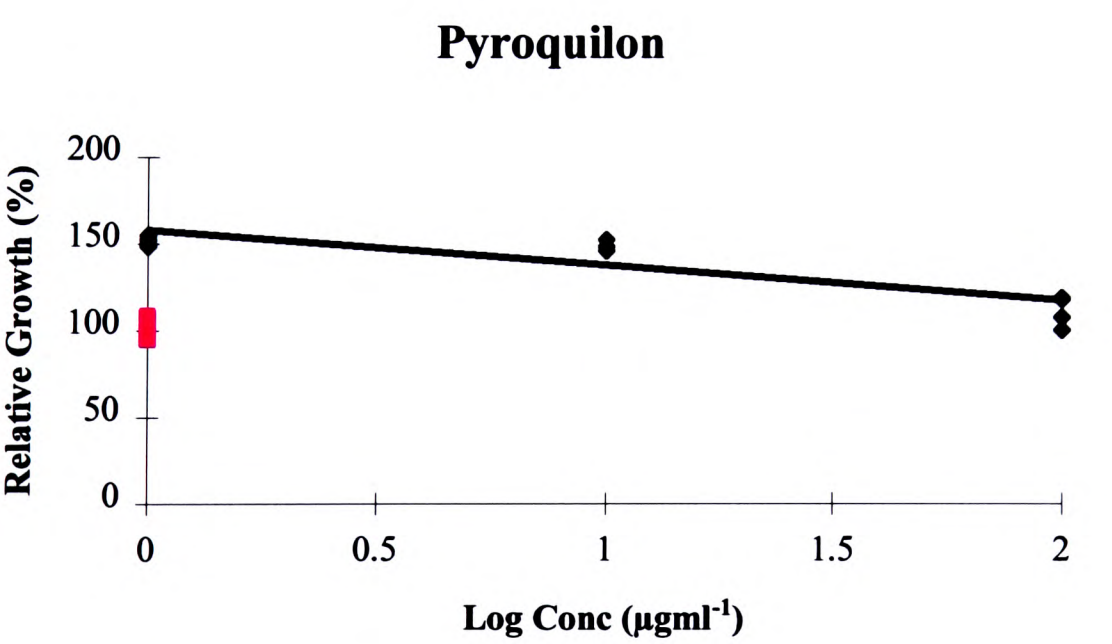


Figure 6.3: Graphs showing the effect of increasing compound concentration on the relative growth of wild-type strain 22-20 after 21 days growth. ■ Controls.

The DOPA-melanin inhibiting compounds slowed down the rate of pigmentation of the colonies but did not alter their colour at any of the concentrations evaluated. The yellow pigmentation was initially seen concurrently with growth before the development of the typical pigment colour of the mutant or wild-type (see Plate 6.6a). Growth was affected in a similar pattern to the DHN-melanin inhibiting compounds with the exception of tropolone and 2-mercaptobenzimidazole which at 100 μg ml^{-1} completely inhibited mycelial growth.

The antibiotic cerulenin did not reduce the colour pigmentation and thus it did not inhibit pentaketide synthesis at either of the concentrations tested in the wild-types or colour mutants listed in Table 6.8. It was found that at 100 μg ml^{-1} the intensity of pigment production was reduced and that patches of darkly pigmented mycelia occurred in the centre of some of the albino colonies. At 100 μg ml^{-1} the wild-type strain C87/631/1 and its colour mutants also showed an altered morphology similar to that of a R-type strain, which can be seen on Plate 6.6b. Mycelial growth at 1 μg ml^{-1} was significantly ($P<0.05$) reduced in some of the wild-types and mutants and at 100 μg ml^{-1} significantly ($P<0.05$) reduced in all the wild-types and mutants.

Table 6.8: The effect of 100 μg ml^{-1} cerulenin on colony colour and morphology of wild-types and mutants after 21 days growth.

Wild-type/Mutant	Colony Colour	Colony Colour with Cerulenin	Morphology with Cerulenin
22-20	Black	Grey/Black	W-type
MT180	Yellow	Yellow	W-type
MT156	Green	Green	W-type
C87/631/1	Black	Grey/Black	R-type
MC87J	Green	Green, black centre	R-type
MC87K	Buff	Buff, black centre	R-type
MC87A	Buff	Buff, black centre	R-type
MC87AA	Albino	Albino, black centre	R-type
C78/501	Dark Brown	Yellow/Brown, black centre	W-type
22-8	Dark Brown	Brown	R-type

6.3.5 Examination for ultra-structural differences using transmission electron microscopy

Examination of the darkly pigmented wild-type strain 22-20 using transmission electron microscopy found that melanin type granules were in the cytoplasm close to the cell wall (Plate 6.8). The cytoplasm was heavily stained making the granules difficult to see. In comparison with the wild-type hyphae (see Plate 6.7a) the albino mutants appeared different in structure with the hyphae having larger vacuoles and a general globular appearance which may be lipid (see Plate 6.7b). Examination where possible of the cytoplasm identified no melanin type granules in the albino. However the cytoplasm was difficult to visualise because it was pushed against the plasmalemma by the very large vacuoles.

6.3.6 Genetic analysis of the colour mutants

Apothecia were produced after 10 months incubation from crosses with the wild-type strain 22-20 and its colour mutants with the wild-type strains C87/631/1, C78/501, C91/761 and 22-433. All apothecia from these crosses were grey/black in colour (Plate 6.9a) and were found both on the nodes of the barley straw and on the bits obtained from the mushroom compost placed on the HP agar. Table 6.9 shows the crosses in which apothecia were produced. Wild-types C87/631/1 and C78/501 were the most fertile when crossed with 22-20 and its colour mutants with most apothecia being seen from these crosses. The green (MT156) and buff (MT38) colour mutants did not produce any apothecia with any of the wild-type strains. Control plates also produced no apothecia.

Table 6.9: Apothecia production from wild-type strain 22-20 and its colour mutants when crossed with wild-type strains C87/631/1, C78/501, C91/761 and 22-433.

Wild-type/Colour Mutant		**C87/631/1**	**C78/501**	**C91/761**	**22-433**
	Mycelial Colour	Black	Brown/Black	Brown	Black
22-20	Black	+	+	-	+
MT178	Albino	+	+	-	+
MT485	Albino	+	+	-	-
MT183	Albino	+	+	-	+
MT141	Albino	+	+	-	+
MT77	Albino	+	+	-	+
MT38	Buff	-	-	-	-
MT88	Yellow	+	+	-	+
MT180	Yellow	+	+	-	-
MT156	Green	-	-	-	-
MT215	Brown	+	+	+	+

+ Apothecia produced
- No apothecia produced

Crosses with strain 22-20 and its colour mutants with strain C87/631/1 and its colour mutants produced some apothecia after 8 months incubation. Where apothecia were produced they varied in colour either being grey/black or buff depending upon the cross and can be seen in Table 6.10, Plate 6.9b.

Table 6.10: Apothecia colour from combinations of colour mutants and wild-types.

Sexual Cross	**Apothecia Colour**
22-20 (black) x C87/631/1 (black)	Grey/Black
22-20 (black) x MC87A (buff)	Grey/Black
22-20 (black) x MC87D (buff)	Grey/Black
C87/631/1 (black) x MT183 (albino)	Grey/Black
MC87J (green) x MT183 (albino)	Grey
MC87C (buff) x MT183 (albino)	Buff
MC87A (buff) x MT485 (albino)	Buff
MC87C (buff) x MT485 (albino)	Buff

Random sampling of the ascospores from selected apothecia produced different phenotypes varying in pigmentation colour and colony morphology. These are summarised in Table 6.11 and Plate 6.9c. Generally crosses with the albinos MT183, MT485, MT178, MT77 and MT141 and the black wild-type strains C87/631/1 and C78/501 produced either albino or brown/black progeny in 1:1 ratios respectively. Crosses with the yellow mutants MT180 and MT88 with the black wild-type C87/631/1 produced yellow, brown, cream and buff progeny, with over half of the progeny being brown in each case. Crosses with the brown mutant MT215 and black wild-type strain 22-20 with black wild-type strains C87/631/1 and C78/501 produced only brown/black progeny. Colony morphology of the progeny was either that of a W-type or combination of a W and R-type. Both colony morphology and pigment intensity varied for each of the progeny and appeared to be independent of its parents.

Table 6.11: Progeny colour from ascospores of different crosses.

Black x Albino

Cross	**Progeny Number Brown**	**Progeny Number Albino**	**Other progeny colour (number)**
C87/631/1 (black) x MT183 (albino)	35	34	
C87/631/1 (black) x MT77 (albino)	38	32	
C78/501 (black) x MT77 (albino)	68	63	
C87/631/1 (black) x MT141 (albino)	36	14	
C78/501 (black) x MT141 (albino)	20	11	green (2)
C87/631/1 (black) x MT178 (albino)	12	14	
C78/501 (black) x MT178 (albino)	61	71	buff (6) yellow (1)
C87/631/1 (black) x MT485 (albino)	40	35	

Black x Yellow

Cross	Progeny colour and number			
	Brown	**Cream**	**Yellow**	**Buff**
C87/631/1 (black) x MT88 (yellow)	33	14	14	1
C87/631/1 (black) x MT180 (yellow)	34	6	3	0

Black x Brown/Black

Cross	Progeny colour and number			
	Brown	**Green**	**Yellow/ Green**	**Black**
C87/631/1 (black) x MT215 (brown	6	2	5	1
C87/631/1 (black) x 22-20 (black)	21	4	0	0
C78/501 (black) x 22-20 (black)	100	0	0	24

6.3.7 Pathogenicity of the colour mutants

Differences in pathogenicity were found between the wild-type strains and the colour mutants when the control plants were excluded. The controls strains 22-20 and C87/631/1 showed high levels of pathogenicity. Thus indicated conditions were suitable for disease development. The control plants showed no disease symptoms. The differences between pathogenicity of the colour mutants could not be directly related to mycelium colouration *in vitro*. Statistical analysis of the data found significant differences ($P<0.05$) between the total set of data, when the un-inoculated controls were excluded and when un-inoculated controls and wild-type strains were excluded. Mutants classed as being pathogenic (having a sum infection score greater than 5 from the 3 pots) were significantly ($P<0.05$) different from each other and from the wild-type strains. No blocking effect was detected when analysed using a two factor ANOVA with replication.

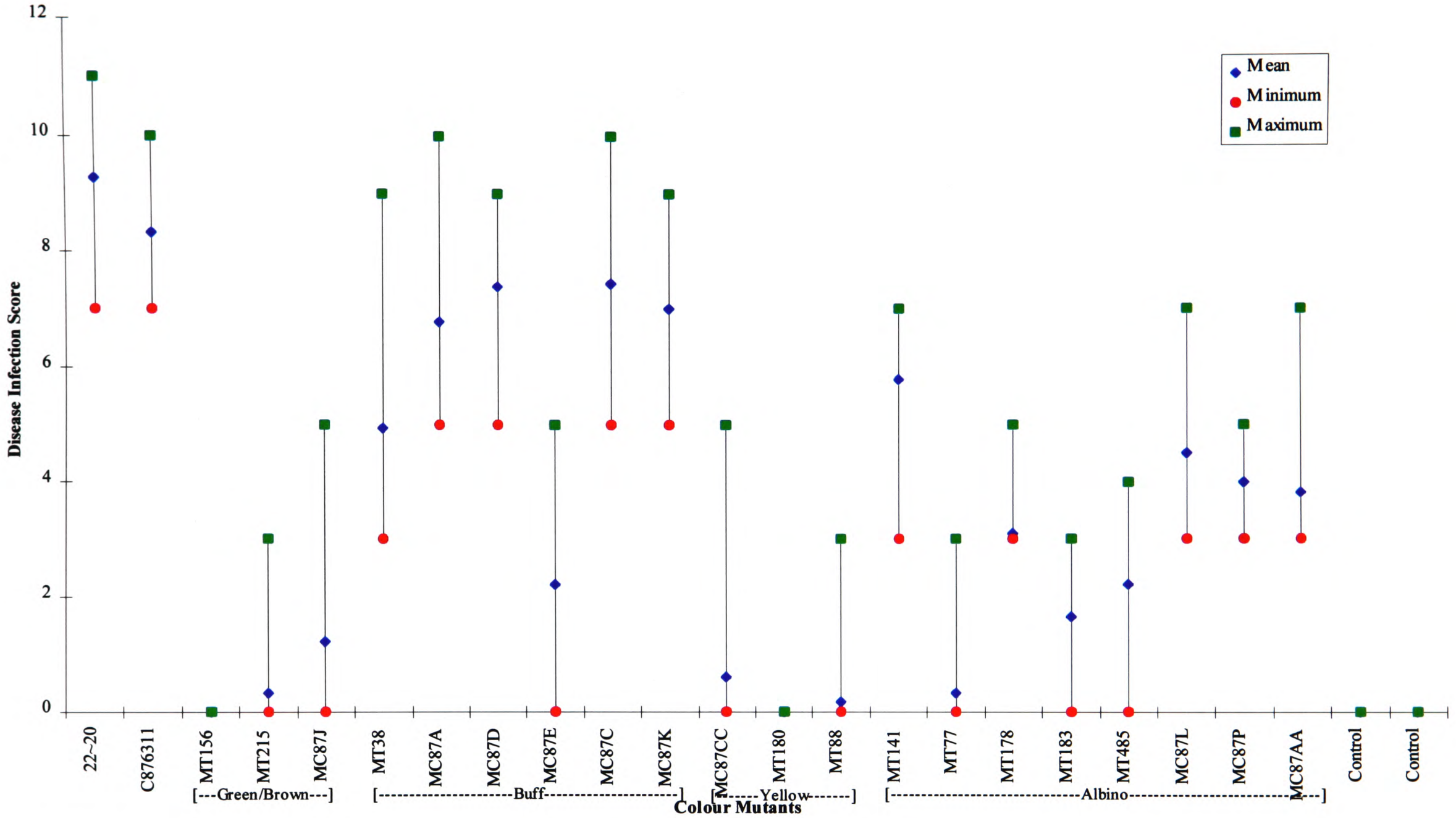


Figure 6.4: Graph showing the pathogenicity of the wild-types and colour mutants inoculated on to wheat seedlings.

Figure 6.4 shows the mean, minimum and maximum disease infection scores of the wild-types and colour mutants inoculated on to wheat. Application of the Tukey Test for non-parametric multiple comparisons using ranked data found that pathogenicity of the colour mutants allowed them to be grouped together. This is seen in Figure 6.4 with the data grouped according to colour and Table 6.12 with the data placed in increasing pathogenicity. From this it can be concluded that the yellow, green and brown mutants were low in pathogenicity, the albino mutants were of higher pathogenicity followed by the buff mutants with the wild-types being the most pathogenic.

Table 6.12: Results of the Tukey Test for multiple comparisons of non-parametric data using ranked data of decreasing pathogenicity of the wild-type and colour mutants inoculated on to wheat. Strains with the same letter are not significantly ($P<0.05$) different.

Colour	Strain												
Black	22-20	a											
Black	C87/631/1	a	b										
Buff	MC87D	a	b	c									
Buff	MC87C	a	b	c	d								
Buff	MC87K	a	b	c	d	e							
Buff	MC87A	a	b	c	d	e	f						
Albino	MT141	a	b	c	d	e	f	g					
Buff	MT38	a	b	c	d	e	f	g	h				
Albino	MC87L	a	b	c	d	e	f	g	h	i			
Albino	MC87P		b	c	d	e	f	g	h	i	j		
Albino	MC87AA		b	c	d	e	f	g	h	i	j	k	
Albino	MT178			c	d	e	f	g	h	i	j	k	l
Albino	MT485							g	h	i	j	k	l
Buff	MC87E							g	h	i	j	k	l
Albino	MT183								h	i	j	k	l
Green	MC87J								h	i	j	k	l
Yellow	MC87CC										j	k	l
Albino	MT7											k	l
Brown	MT215											k	l
Yellow	MT88												l
Green	MT156												l
Yellow	MT180												l
	Control												l

The pathogenicity results indicate that melanin is not a specific pathogenicity determinant as the albino mutants showed moderate and buff mutants showed high levels of pathogenicity.

Significant ($P<0.05$) differences were found between strain and colour mutants in the number of leaf sheaths on which infection plaques were seen. This was expected as the depth of the disease symptoms varied in extent through the stem bases. Infection plaques were found to be present on leaf sheaths or a leaf sheath in advance of the disease symptoms, following a predicted pattern of fungal invasion in advancement of symptom expression (as described in chapter 5). Previous studies described in chapters 4 and 5 using the fluorochrome ANS to stain hyphal structures found that at the centre of large infection plaques some cells didn't appear to fluoresce as brightly as the surrounding cells. This was attributed to pigments being present in these cells. It was noted however in the present experiment that stained darker cells were also present in the infection plaques of albino mutants. Although a study was not made on melanin production when the pathogen is colonising the host, no melanin had ever been visualised from albino mutants grown *in vitro*.

All pathogenic strains were able to form W-type (spreading) infection plaques which tended to be seen at the edge of necrotic lesions. The infection plaques varied in size with the albino, yellow, green and brown mutants generally seen to produce smaller infection plaques than the buff and wild-types. Runner hyphae were recorded on most samples where host colonisation occurred. Of the mutants classed as non-pathogenic only the brown mutant MT215 was found to produce infection plaques on the 1st leaf sheath. The presence of these infection structures indicates that melanin does not play a role in their formation. All colour mutants were less pathogenic than the wild-types.

6.3.8 The effect of oxidation on the wild-types and colour mutants

Addition of sodium hypochlorite to the colonies growing on MYG agar and cellophane discs oxidatively bleached them, the end point being a totally white colony and can be seen in Plate 6.10. Two different strengths of sodium hypochlorite were used, 4% sodium hypochlorite was added directly to the colonies growing on MYG agar and 1% to the colonies growing on cellophane discs. This was because colonies growing on MYG agar had excreted pigments into the media and there was a larger volume of pigments to be bleached compared to only the pigments present in the hyphae growing on the cellophane discs. Significant differences ($P<0.05$) were found between the times taken to bleach the colonies growing both on cellophane discs and MYG agar. Figure 6.5 shows the mean and standard error of the times taken for bleaching to occur on colonies growing on cellophane discs. Bleaching times of colonies grown on MYG agar were approximately twice those of colonies grown on cellophane.

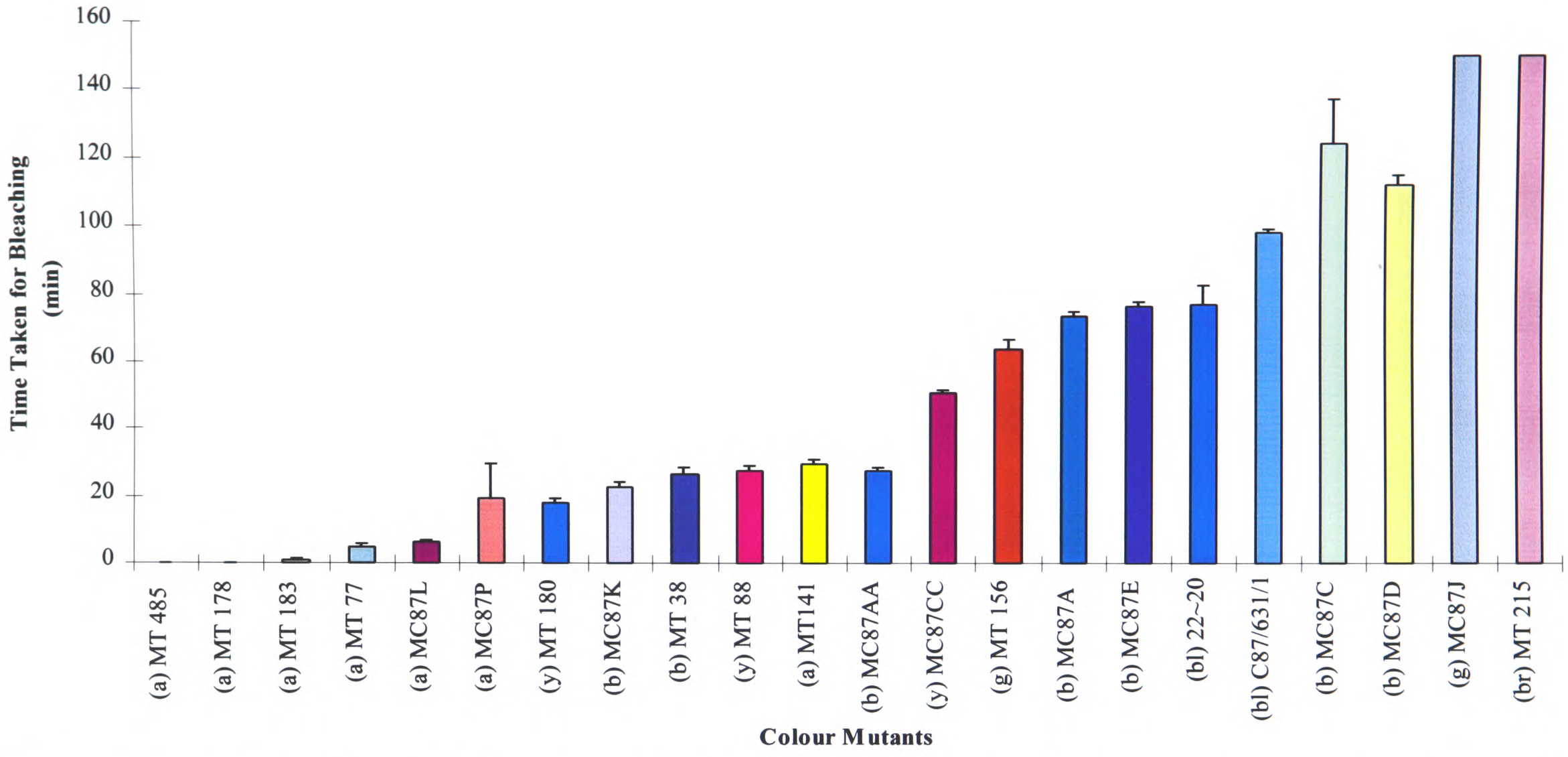


Figure 6.5: Graph showing the average time taken to bleach fungal colonies grown on cellophane discs using 1% sodium hypochlorite. Error bars represent the standard error for each mean. a-Albino, b-Buff, y-Yellow, bl-Black, br-Brown, g-Green.

The time taken to bleach the albino colonies was between 0-6 minutes, the yellow colonies took 10-30 minutes, buff colonies took 20-125 minutes and brown, green and black colonies 65-150 minutes. Differences were seen within the coloured mutant classes due to the intensity of pigment production. Two of the buff colour mutants MT38 and MC87K were very pale and thus were bleached quicker than the darkly pigmented buff mutants. Application of the Tukey test showed overlapping sets of similarity of the colonies grown on MYG agar and cellophane. Table 6.13 shows the results of the Tukey Test analysed for the colour mutants grown on cellophane discs.

Table 6.13: Results of the Tukey Test for multiple comparisons of the time taken to bleach the wild-type strains and colour mutant colonies grown on cellophane discs. Strains listed in increasing resistance to bleaching, strains that share the same letter and not significantly ($P<0.05$) different.

Colour	Strain														
Brown	MT215	a													
Green	MC87j	a													
Buff	MC87C		b												
Buff	MC87D			c											
Black	C87/631/1				d										
Black	22-20				d	e									
Buff	MC87E				d	e	f								
Buff	MC87A					e	f	g							
Green	MT156					e	f	g	h						
Yellow	MC87CC								h	i					
Albino	MT141									i	j				
Albino	MC87AA										j	k			
Yellow	MT88										j	k	l		
Buff	MT38										j	k	l	m	
Buff	MC87K										j	k	l	m	n
Albino	MC87P										j	k	l	m	n
Yellow	MT180										j	k	l	m	n
Yellow	MC87L													m	n
Albino	MT77													m	n
Albino	MT183														n
Albino	MT178														n
Albino	MT485														n

From Figure 6.5 and Table 6.13 clear groupings of the colour mutants can be seen in the time taken for oxidative bleaching. Generally, the darkly pigmented wild-types, brown, green and buff mutants took longer to bleach then the yellow and albino mutants. The darker pigment colour mutants having more pigmentation than the wild-types took longer to bleach.

6.3.9 The effect of UV irradiation on the wild-types and colour mutants

The exposure of colonies grown on MYG agar to UV radiation decreased the ability of mycelium to re-grow after irradiation. Re-growth was from the mycelium grown from the hyphal plug after irradiation compared to the mycelium grown from the un-irradiated control plug when placed on MYG agar. The initial time course found that minimum times of 4 and 15 minutes were required before any inhibition of mycelial growth was seen in the albino and buff mutants MC87AA and MC87A respectively. Increasing the irradiation dose was additionally found to dry up the agar plugs and after 150 minutes irradiation, the albino mutant was killed (see Appendix 6.1). From these initial results 4 irradiation times of 25, 50, 75 and 100 minutes were chosen as this would show the effect of increasing the UV dose on mycelial re-growth without killing the hyphae. For all 4 irradiation doses tested, mycelial re-growth was quicker from the wild-types and pigmented colour mutants compared with the albino mutants (see Plate 6.11a). Figure 6.6 shows the mean re-growth of a selection of wild-types and colour mutants after irradiation for 100 minutes. It can be seen that over the 168 hours incubation period, re-growth of some of the wild-types and colour mutants reached that of the un-irradiated controls. The darkly pigmented and yellow strains showed re-growth of between 5 and 30% after 24 hours, of the albino mutants two showed re-growth of 3% after 48 hours and a further two showed no re-growth until 96 and 144 hours incubation.

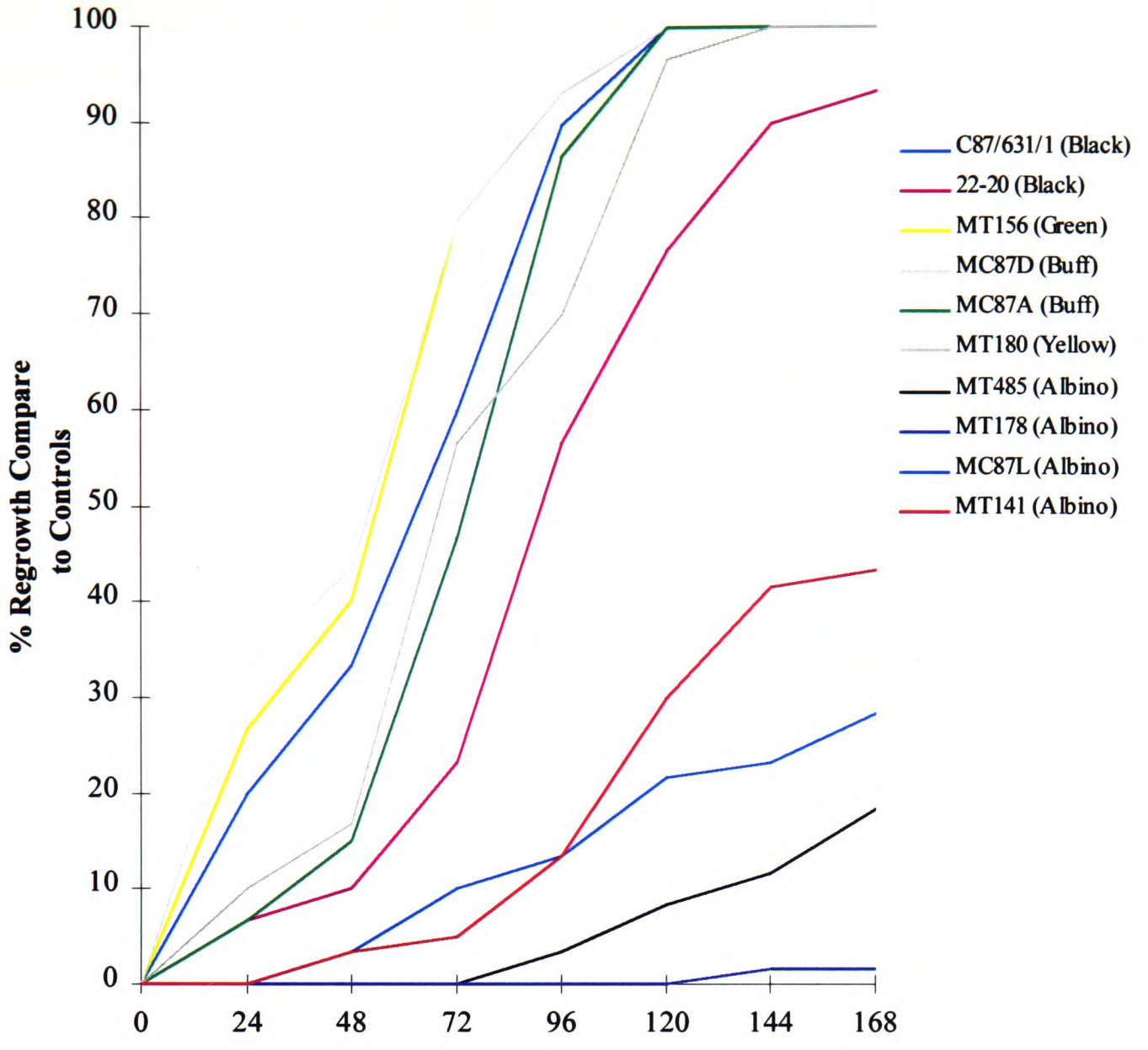


Figure 6.6: The effect of a 100 minute dose of UV irradiation on the re-growth of a selection of wild-types and colour mutants over time.

After all 4 doses of irradiation, mycelial re-growth followed a typical growth curve pattern with an initial lag phase, an exponential phase followed by a stationary phase where growth was completely equal to that of the controls. The curves show different lengths of lag phase with the albino mutants having a longer lag phase compared to the wild-types and pigmented mutants. This is probably due to a greater destruction of cells by the UV irradiation thus taking the hyphal cells which survived irradiation in the centre of the plug longer to grow out.

Rates of re-growth of the wild-types and colour mutants were determined from the exponential phase of growth. Figure 6.7 shows the rates of re-growth of the wild-

type strain 22-20 and its albino mutant MT178 after the varying irradiation doses. It can be seen by the near parallel regression lines that the growth rates (slopes of the regression lines) after leaving the lag phase do not depend on the irradiation dose. This was found to be the case with all of the wild-types and colour mutants tested. The rates of re-growth were greater for the wild-type, brown, green and buff mutants compared to the albino mutants which showed much lower rates of re-growth. The variation encountered in the rates of re-growth corresponded to the presence and extent of pigmentation (the more pigment the faster the re-growth).

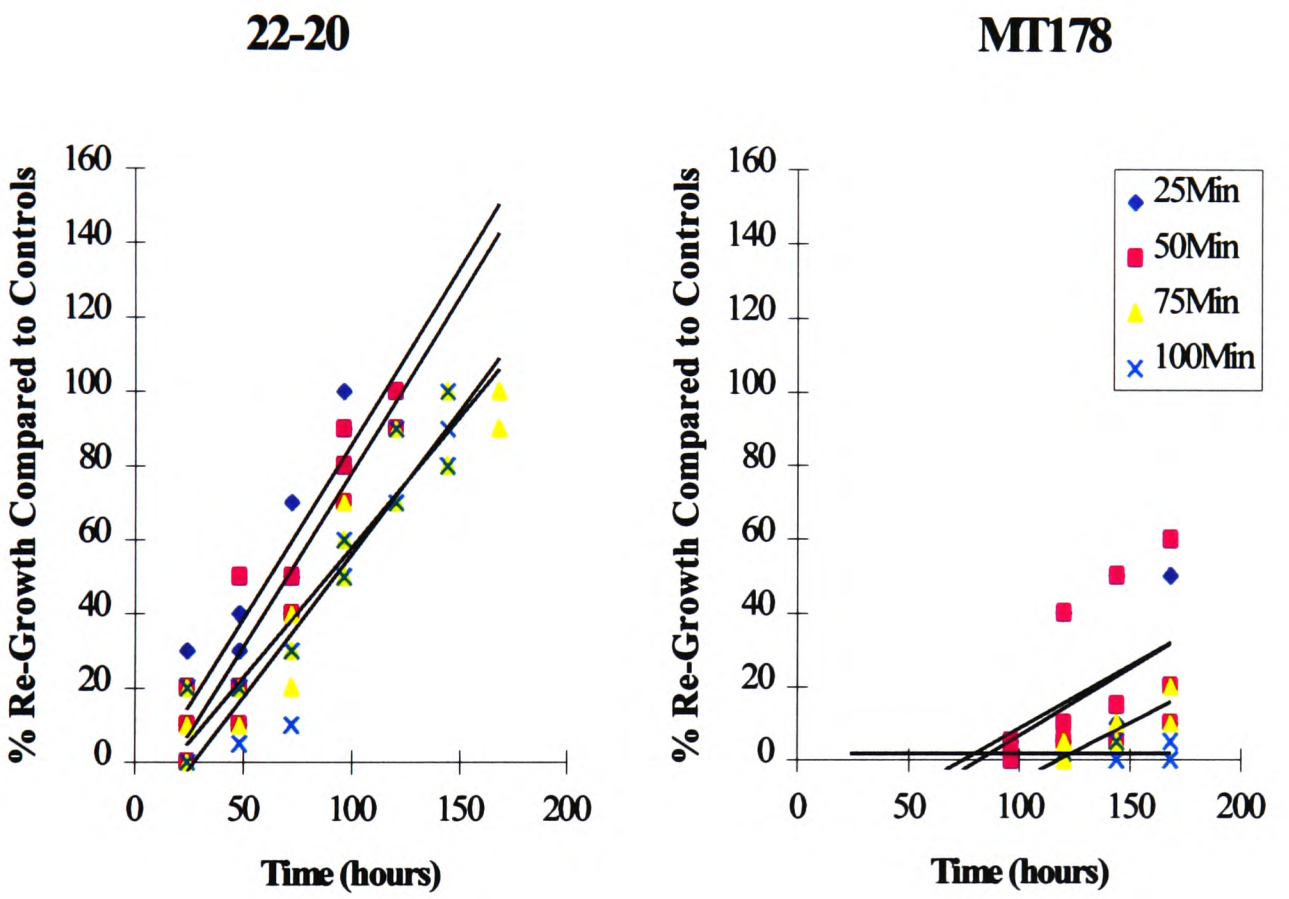


Figure 6.7: Linear regression analysis of the linear growth phase of re-growth in wild-type strain 22-20 and its albino mutant MT178 following increasing irradiation doses.

Increasing the irradiation dose was found to increase the lag phase regardless of colour pigmentation. Figure 6.8 shows the effect of increasing the irradiation dose on the wild-type strain 22-20 and its albino mutant MT178. It can be seen that 24 hours after irradiation the re-growth of strain 22-20 was 4 times greater when it was irradiated for 25 min compared to 100min. In contrast, after 168 hours since

irradiation the re-growth of the albino mutant MT178 was 25 times greater than when irradiated for 25 minutes compared with 100 minutes.

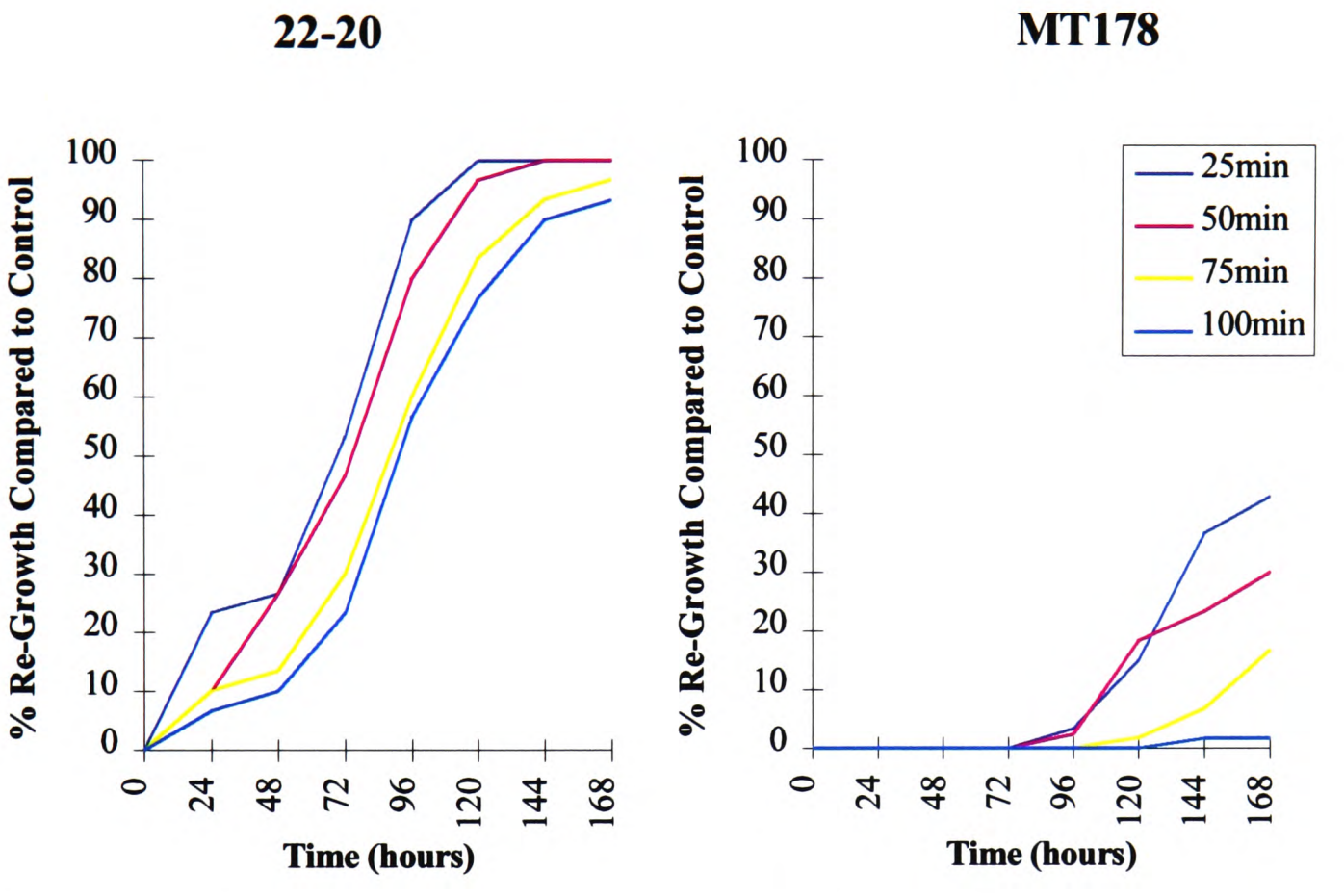


Figure 6.8: The effect of increasing the UV irradiation dose on the re-growth of wild-type strain 22-20 and its albino mutant MT178.

These results show that the presence of pigments increases mycelial survival in the presence of UV irradiation. The degree of protection of melanin can be related to its capacity to undergo reversible changes in free radical content and this appears to be proportional to its concentration in the mycelium and agar.

6.3.10 The effect of desiccation on the wild-types and colour mutants

Desiccation of the colonies decreased their ability to re-grow when placed on fresh media. Desiccation caused the mycelia to shrink away from the edges of the cellophane and the cellophane curled upwards at the edges. The initial time course using wild-types strain C87/631/1 and colour mutants MC87J, MC87A and MT178 showed an almost linear decrease in fresh weight with increasing desiccation time, a

constant dry weight was reached between 30 and 60 minutes desiccation (see Appendix 6.2). Desiccation times of 120 and 360 minutes were thus chosen to examine the mycelial survival when they were desiccated to a constant dry weight. Re-growth was quicker from the wild-types and pigmented colour mutants compared to the albino mutants after both desiccation times, see Plate 6.11b. Figure 6.9 shows the re-growth of a selection of strains after desiccation for 360 minutes. It can be seen that the curves are similar to the growth curves identified after UV irradiation with a lag phase, exponential linear growth phase and stationary phase. Over the incubation period after desiccation some of the pigmented strains re-grew equal to the un-desiccated controls. The darkly pigmented strains showed up to 30% re-growth after 24 hours whereas the albino strains required 48-96 hours incubation before any re-growth was seen. The presence of pigments thus appears to decrease the lag phase for recovery after desiccation.

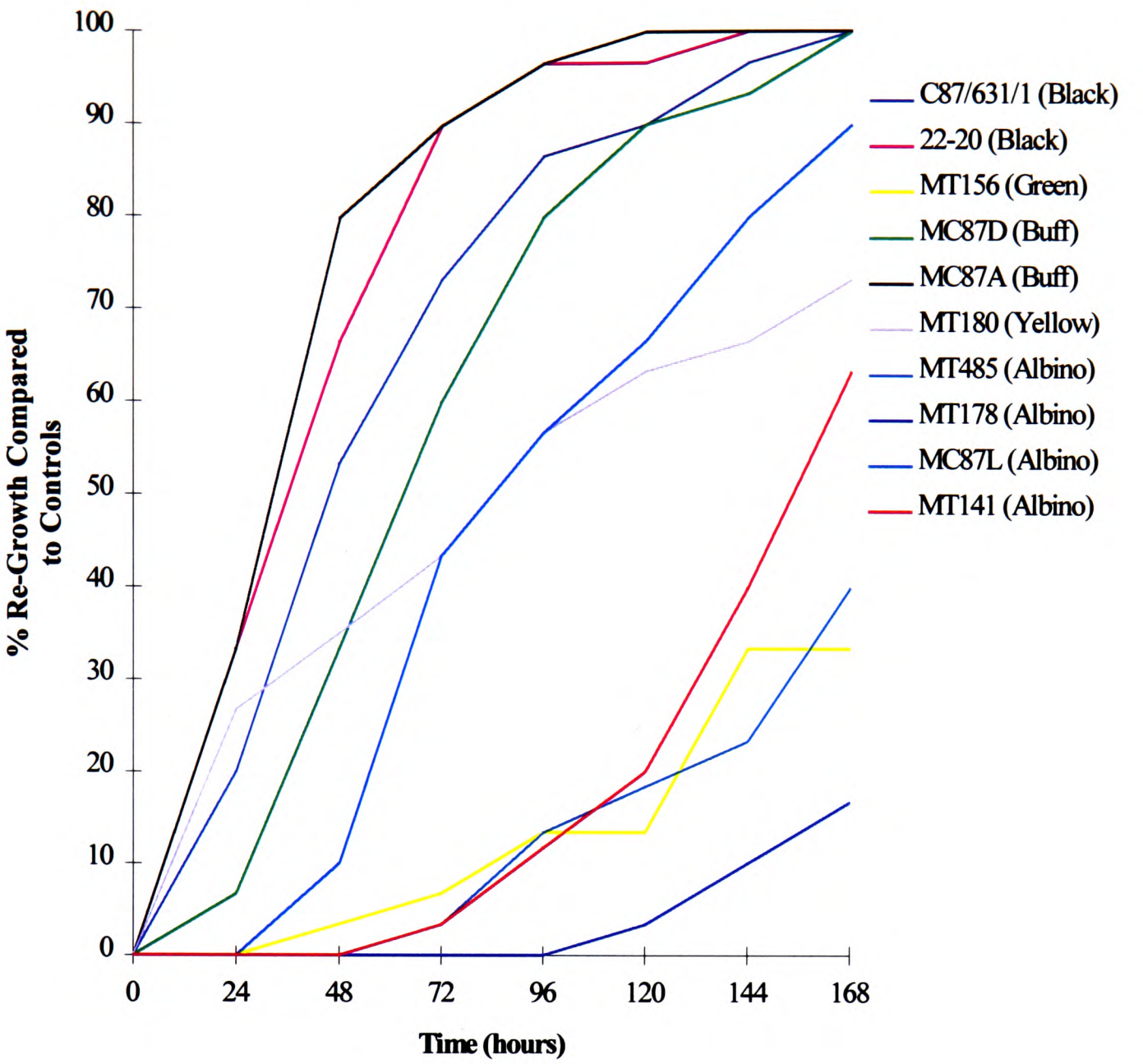


Figure 6.9: The effect of desiccation for 360 minutes on the re-growth of a selection of wild-types and colour mutants over time.

Application of a linear regression analysis to the linear growth phase determined the growth rates. Figure 6.10 shows the growth rates of the wild-type strain 22-20 and its albino mutant MT178. It can be seen by the near parallel regression lines that growth rates were similar regardless of the desiccation time. The growth rates were greater for the pigmented strains compared to the albino mutants. Again variation in rates could be related to the proportion of pigmentation.

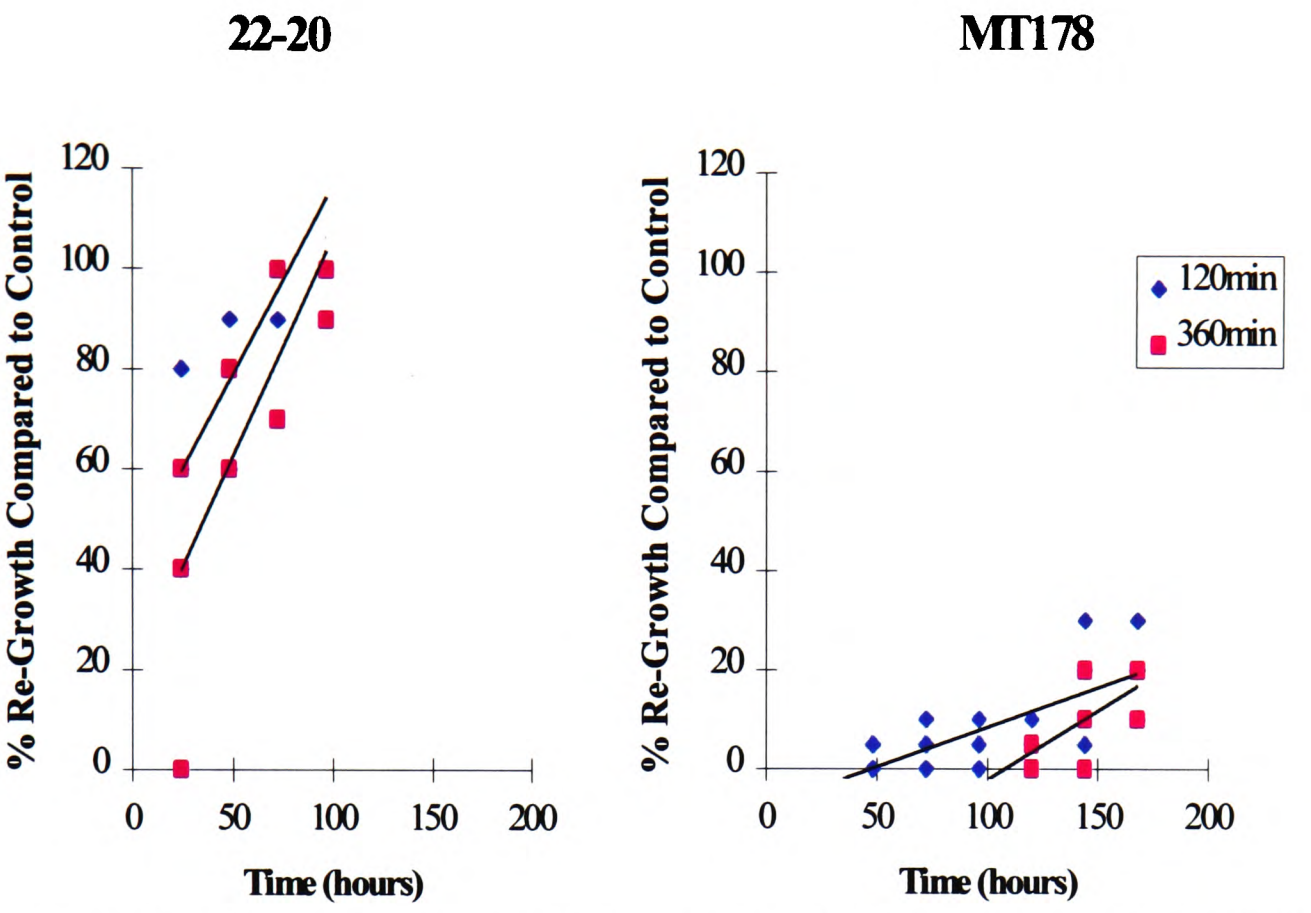


Figure 6.10: Linear regression analysis showing the rates of re-growth of wild-type strain 22-20 and its albino mutant MT178 with increasing desiccation times.

Increasing desiccation time from 120 to 360 minutes was found to increase the lag phase on all wild-types and colour mutants. Figure 6.11 shows wild-type strain 22-20 and its albino mutant MT178. Desiccation of 360 minutes increased the lag time of MT178 from 24 hours to 96 hours. Only a slight increase in lag time was seen for the wild-type strain 22-20.

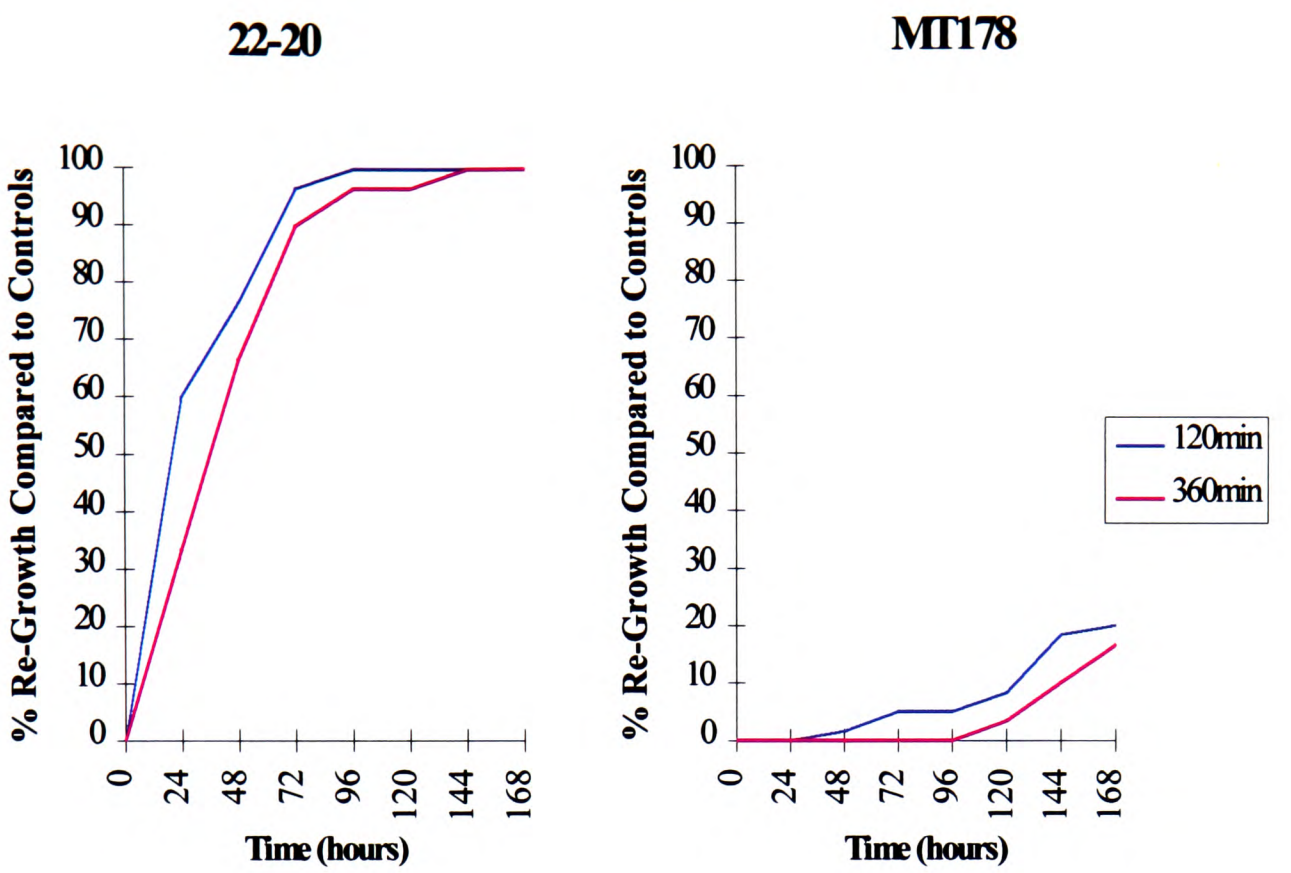


Figure 6.11: The effect of increasing the desiccation time on the re-growth of wild-type strain 22-20 and its albino mutant MT178.

The presence of pigments thus increases survival after desiccation. It is not possible to compare the rates of re-growth with those after UV irradiation because of the different composition of the nutrient medium. The presence of cellophane in desiccation may effect the growth rates of the strains by making nutrients and water less accessible from the agar compared to them grown directly on the agar.

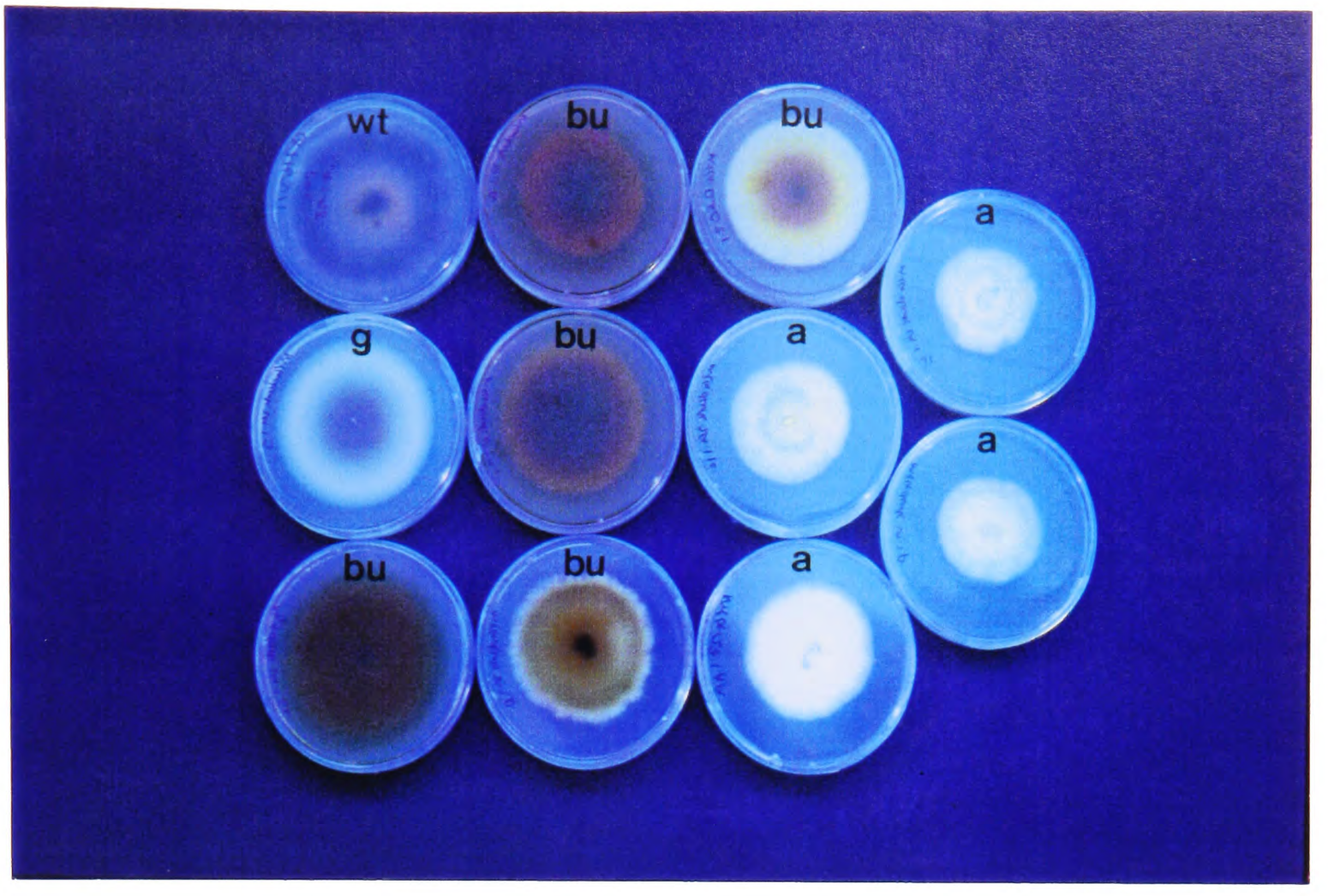
Plate 6.1

Colour mutants produced from *P. herpotrichoides* conidia following UV irradiation.

A) Wild-type strain C87/631/1 (wt) and its, green (g), buff (bu) and albino (a) colour mutants.

B) Wild-type strain 22-20 (wt) and its brown (br), green (g), buff (bu), yellow (y) and albino (a) colour mutants.

A



B

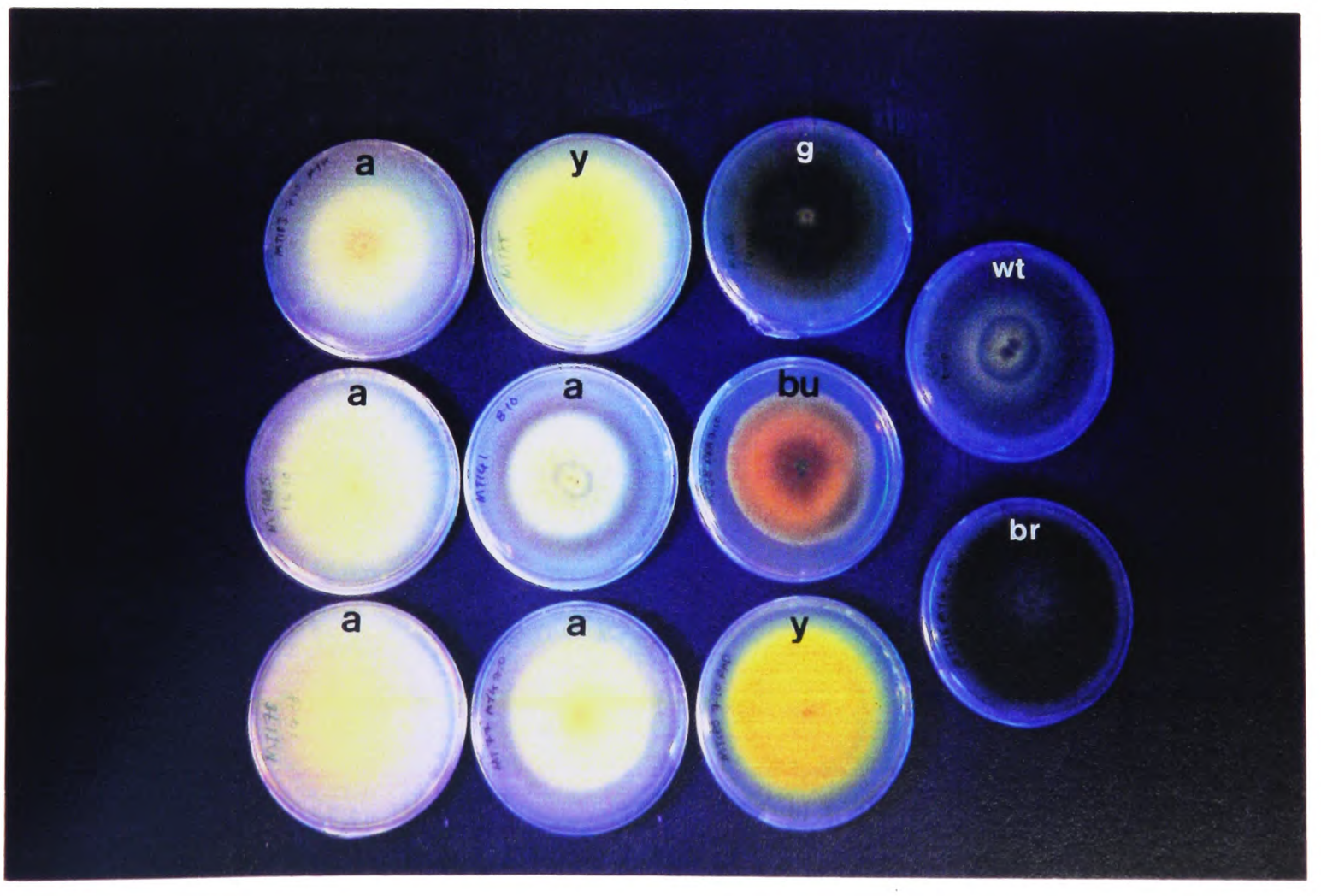


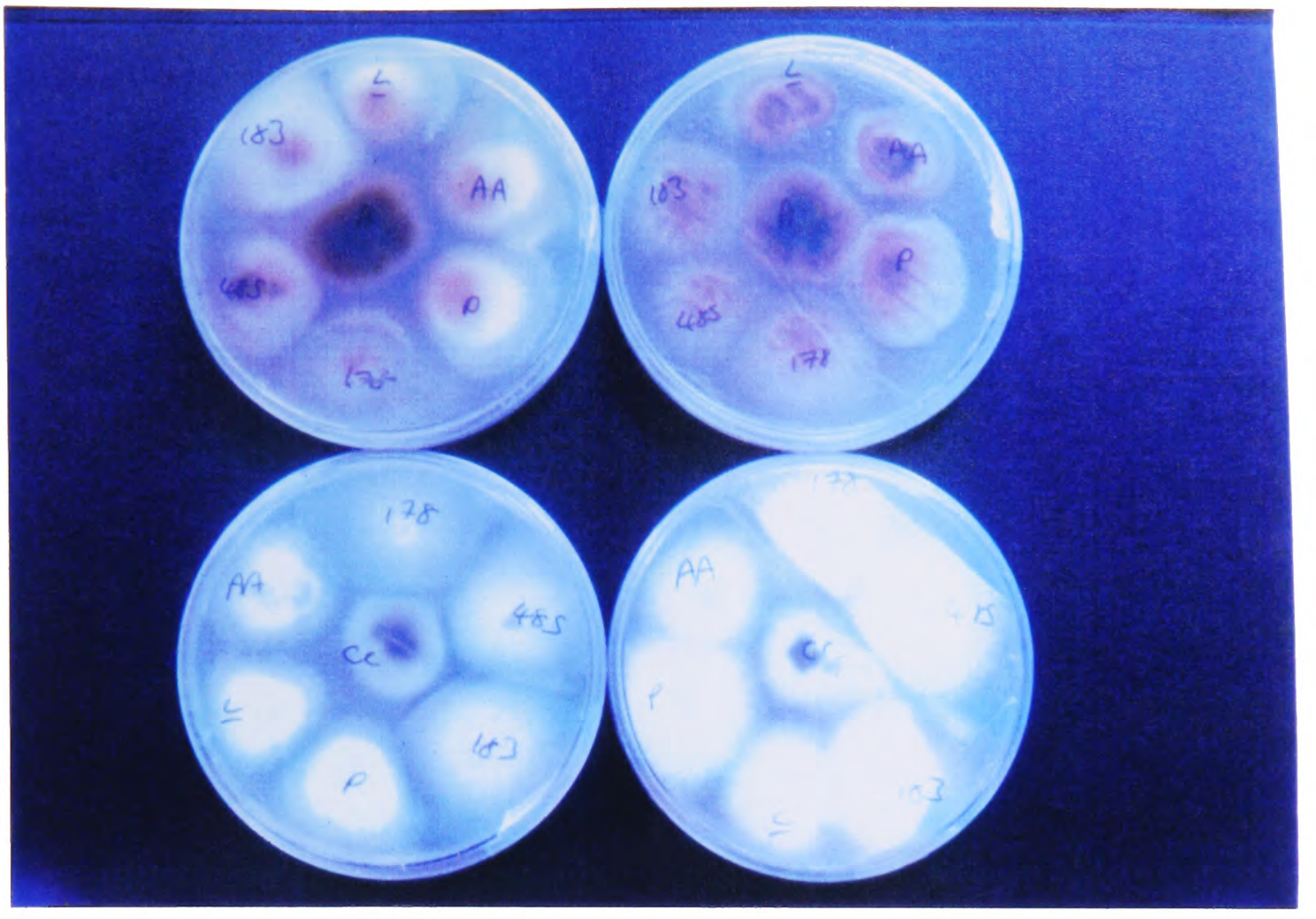
Plate 6.2

Pigment secretion into MYG agar and shake-culture.

A) Secretion of a buff pigment from colour mutant MC87A (A) and its uptake by albino mutants grown on cellophane. Controls inoculated around a non-pigment secreting mutant MC87CC (CC).

B) Pigments released into liquid MYG from wild-type strain C87/631/1 and its colour mutants including an un-inoculated control (c).

A



B

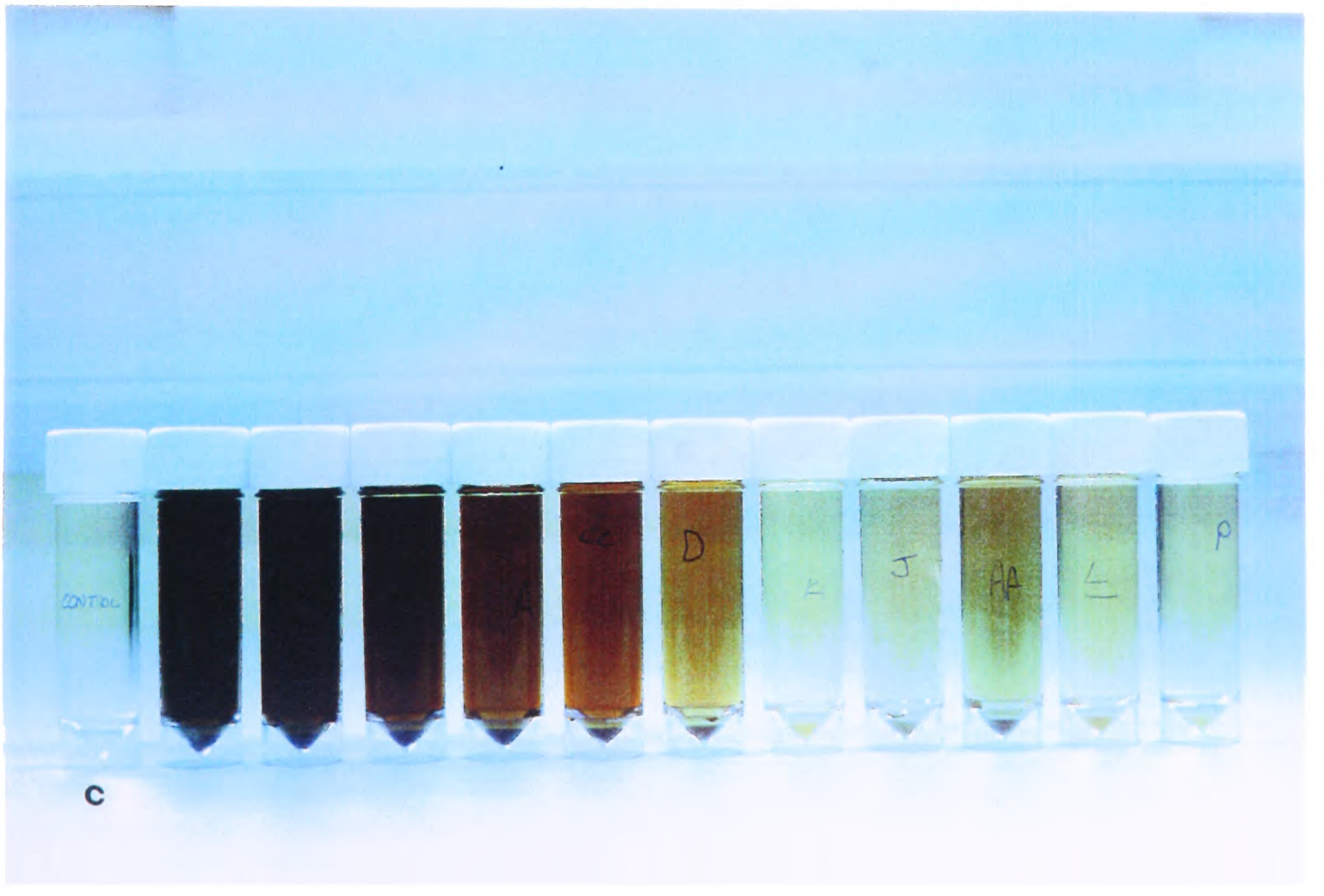


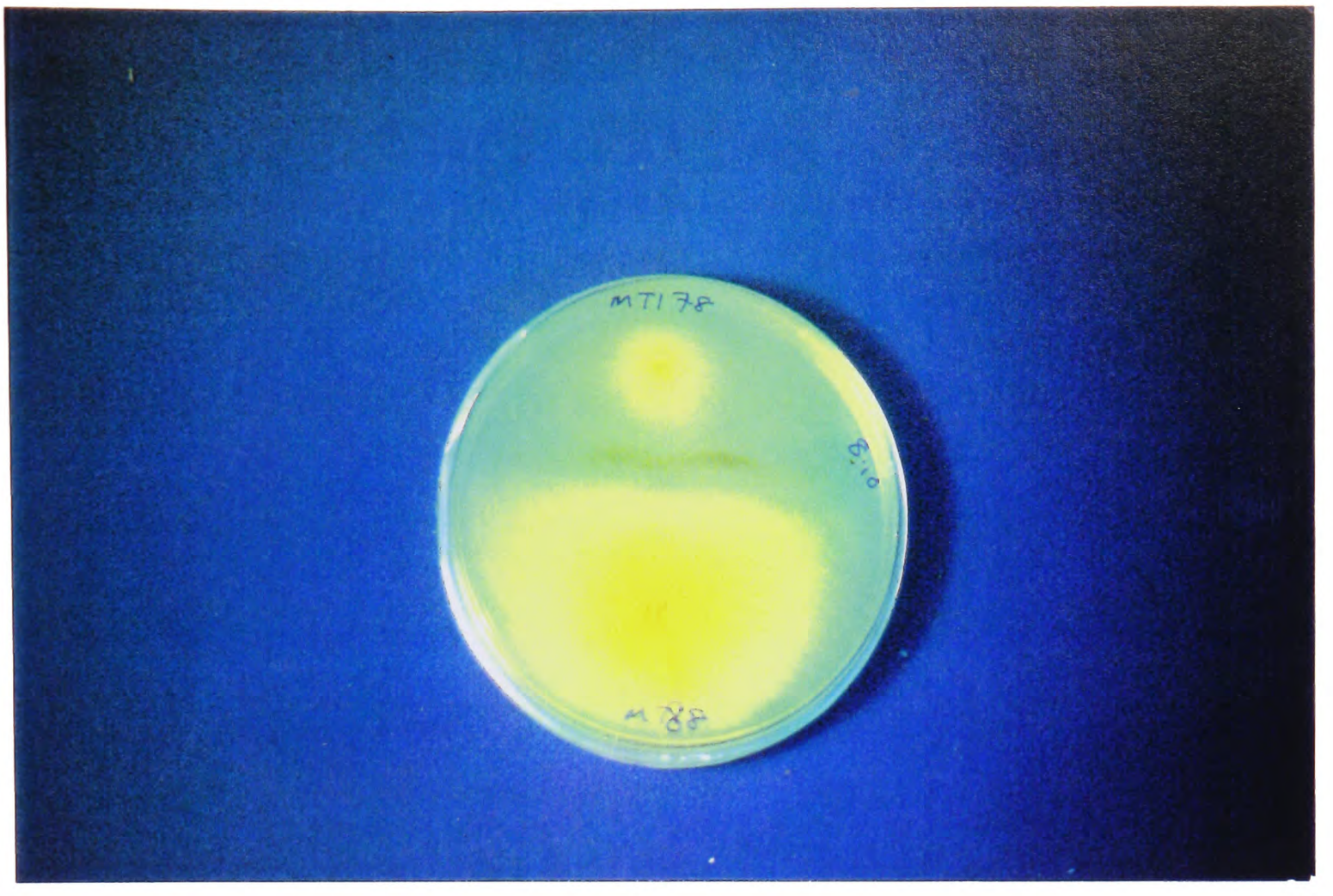
Plate 6.3

Vegetative compatibility parings showing darkly pigmented mycelium at the interface of the colonies.

A) Complementation between colour mutants MT178 and MT88.

B) Complementation between colour mutants MT141 (11) and MT88 (9) inoculated in a grid pattern.

A



B

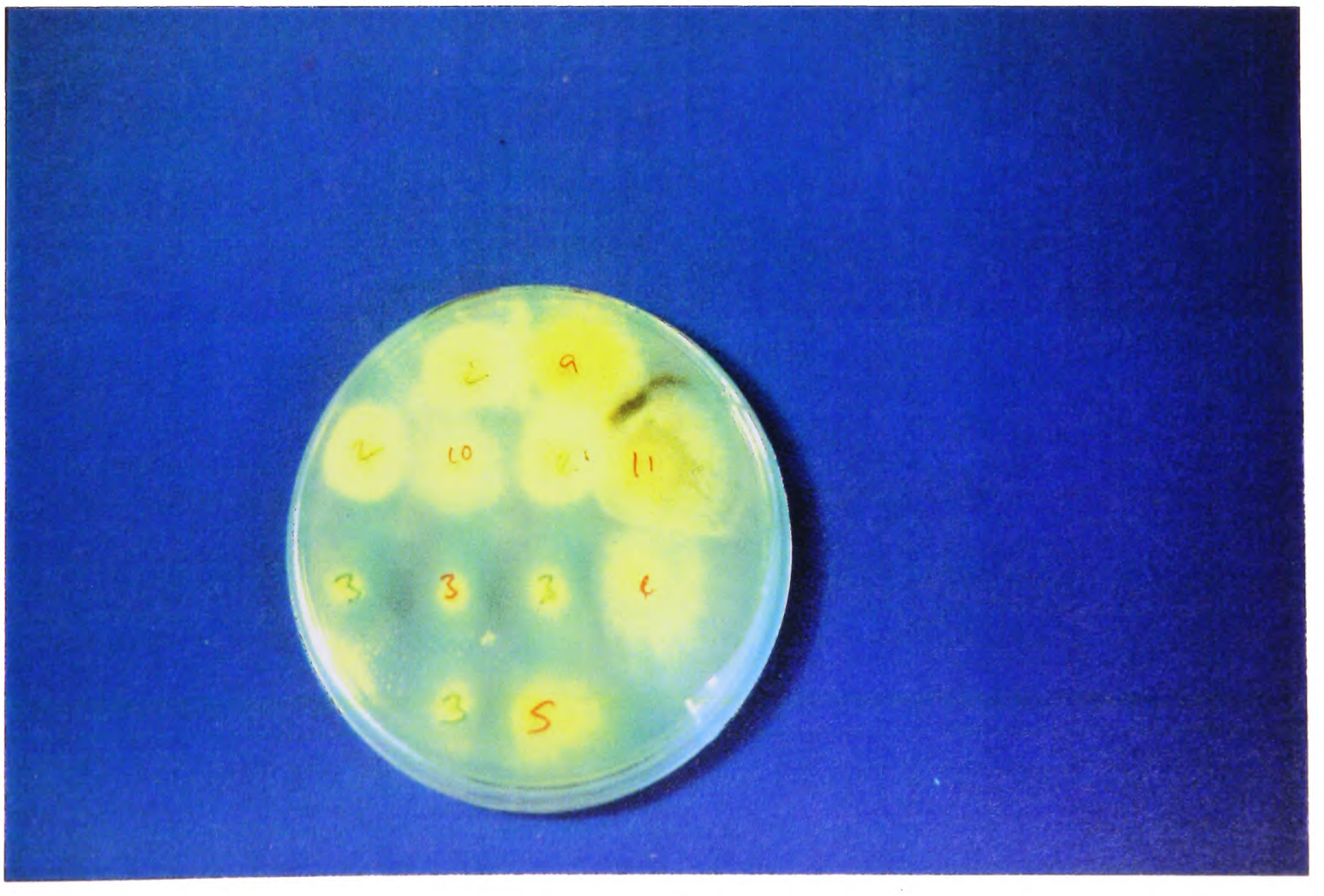


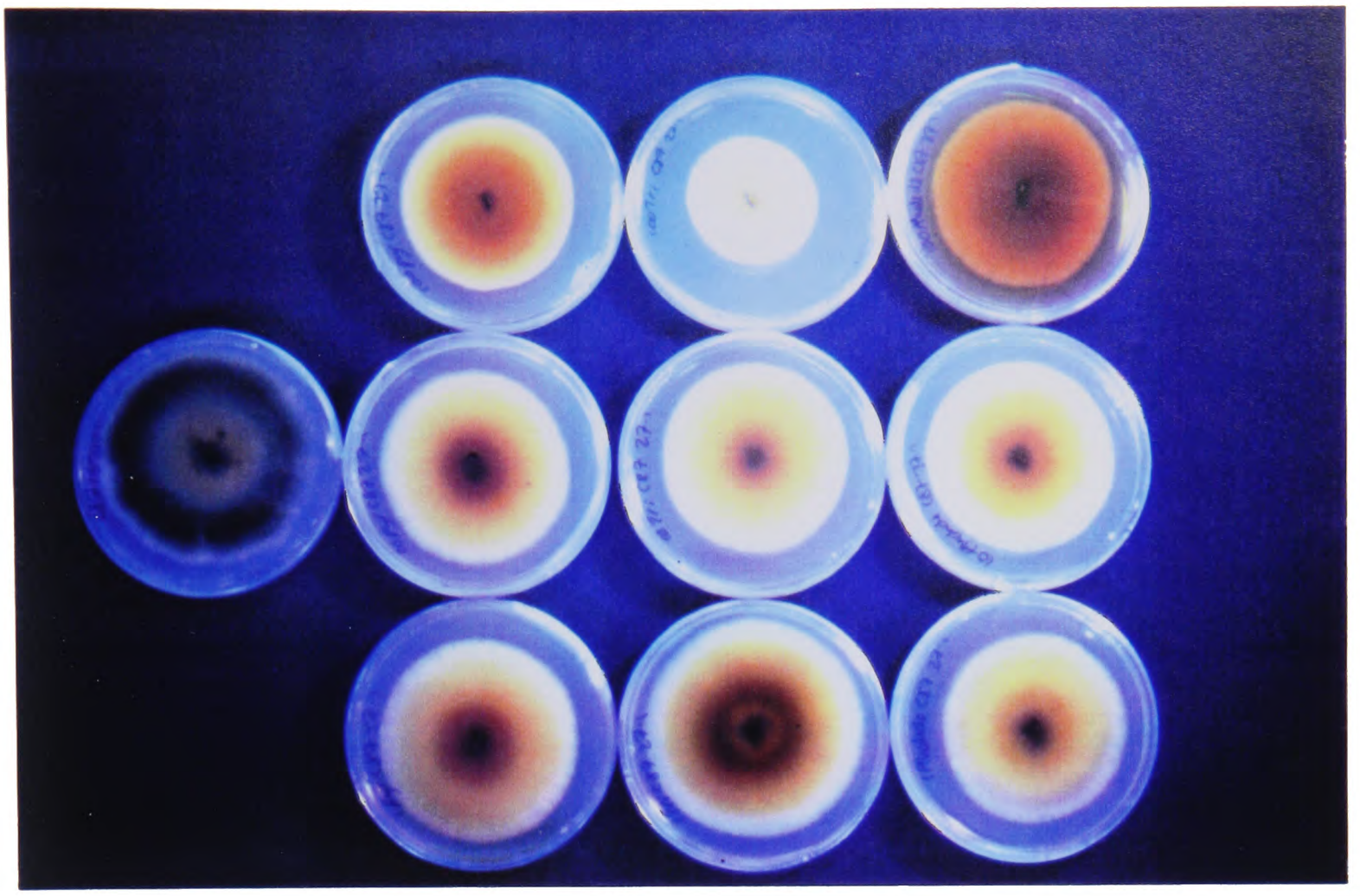
Plate 6.4

The effect of DHN melanin-inhibiting compounds after 21 days incubation at 19°C in the dark.

A) Wild-type strain C87/631/1 grown on MYG containing 1, 10 and 100 $\mu g\ ml^{-1}$ pyroquilon, tricyclazole and fthalide.

B) Buff colour mutant MC87A grown on MYG containing 1, 10 and 100 $\mu g\ ml^{-1}$ pyroquilon, tricyclazole and fthalide.

A



μg ml

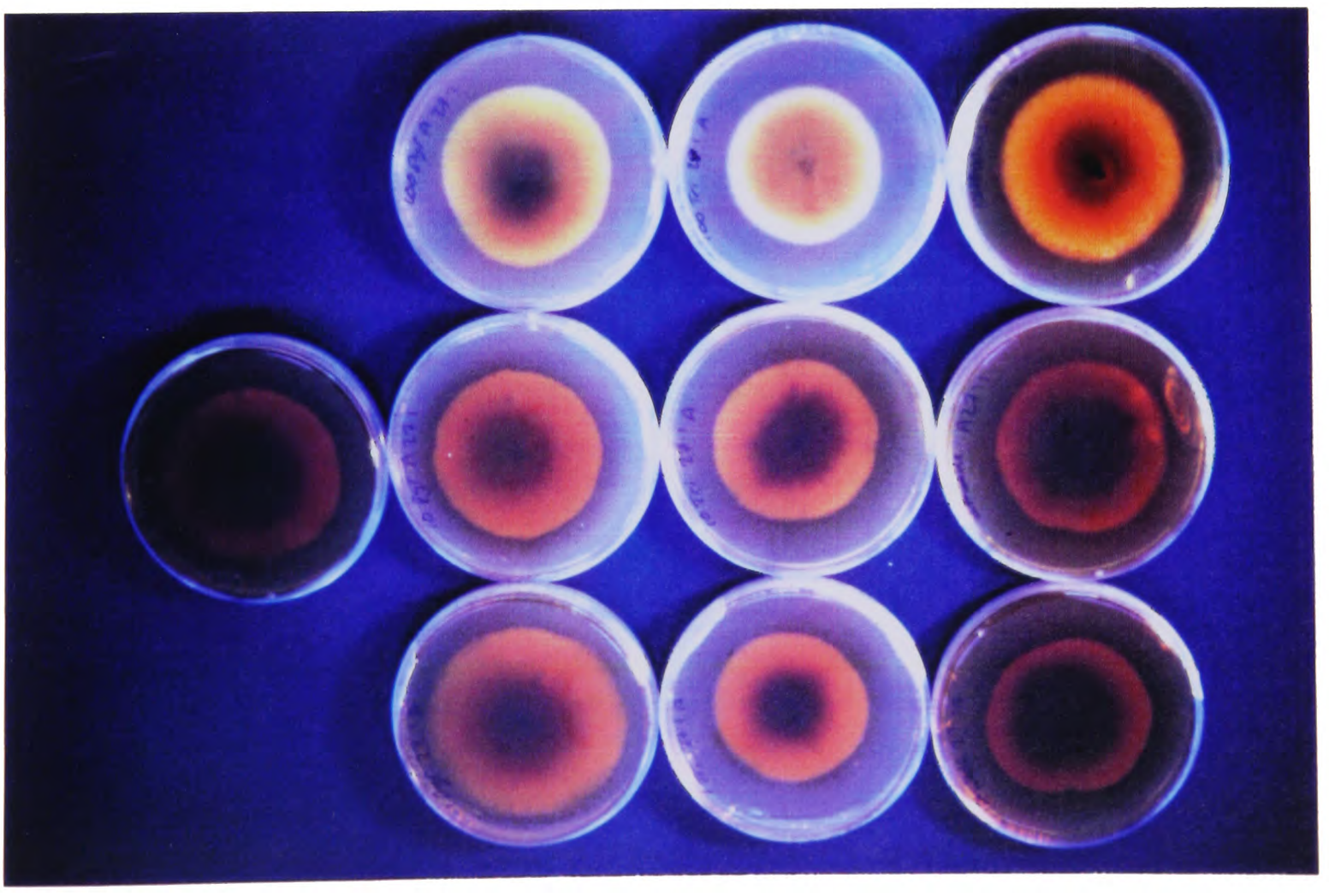
100

10

1

Control Pyroquilon Tricyclazole Fthalide

B



μg ml

100

10

1

Control Pyroquilon Tricyclazole Fthalide

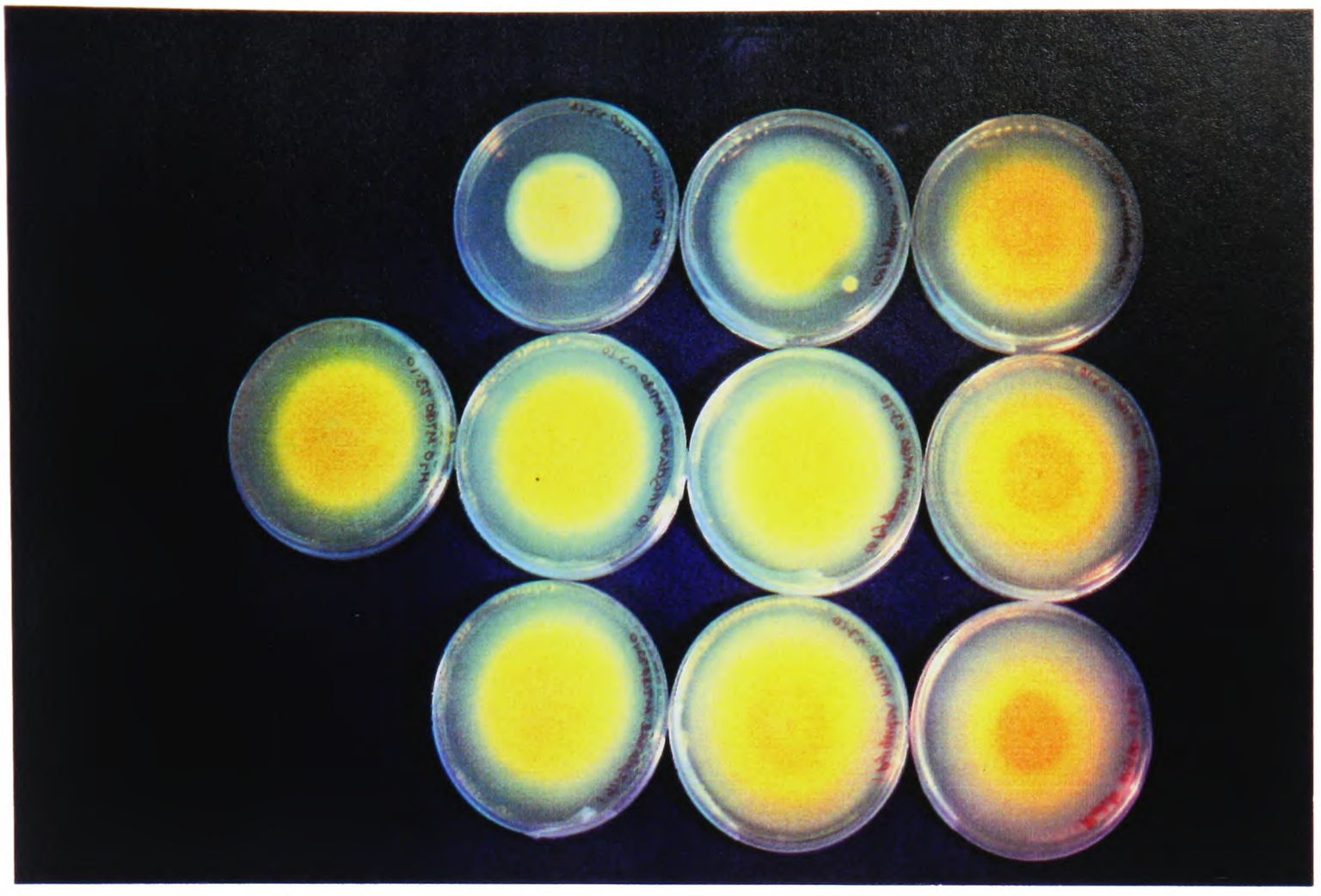
Plate 6.5

The effect of DHN melanin-inhibiting compounds after 21 days incubation at 19°C in the dark.

A) Yellow colour mutant MT180 grown on MYG containing 1, 10 and 100 μg ml^{-1} pyroquilon, tricyclazole and fthalide.

B) Albino colour mutant MT183 grown on MYG containing 1, 10 and 100 μg ml^{-1} pyroquilon, tricyclazole and fthalide.

A


μg ml-
100
10
1
Control
Tricyclazole
Fthalide
Pyroquilon

B

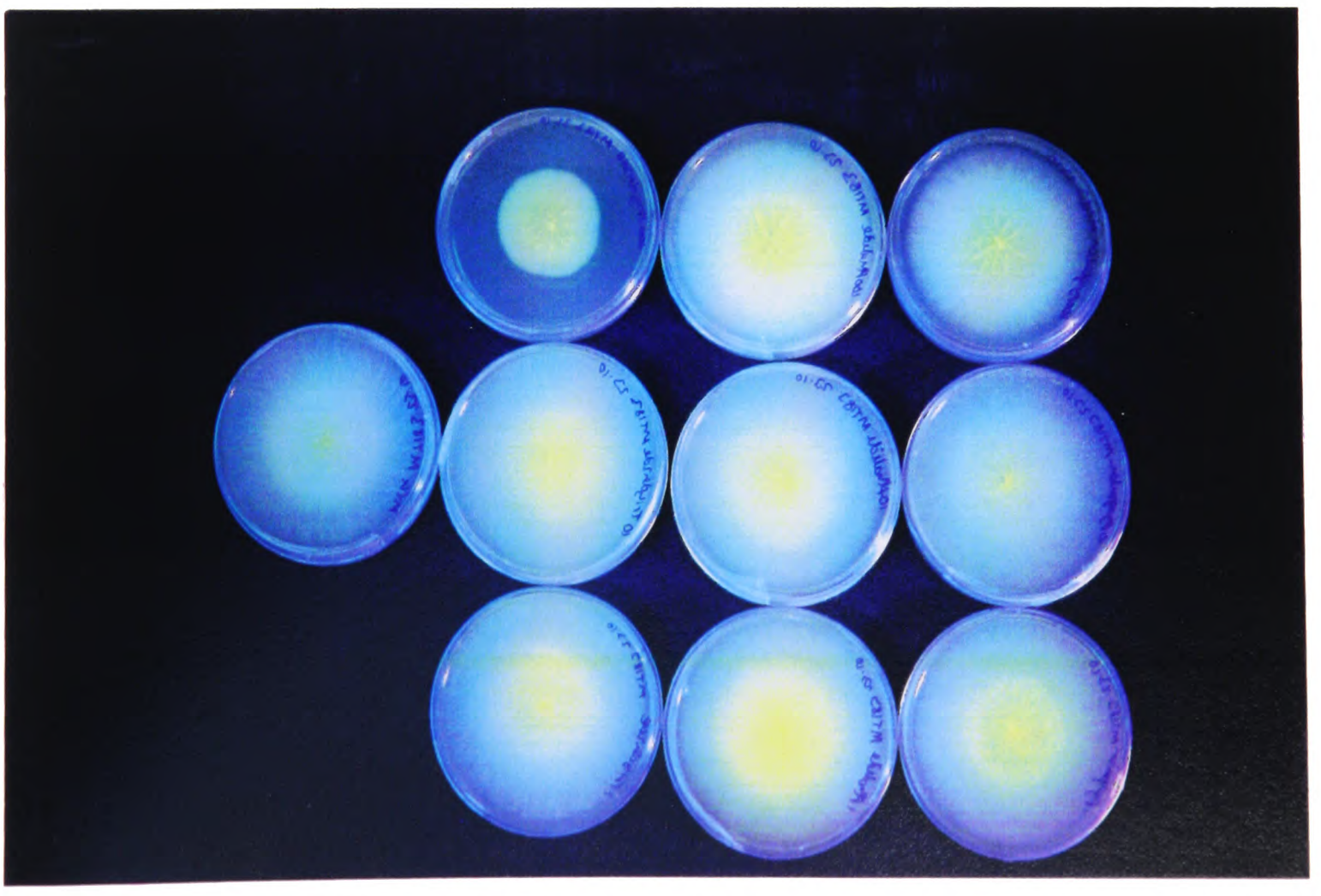

μg ml-
100
10
1
Control
Tricyclazole
Fthalide
Pyroquilon

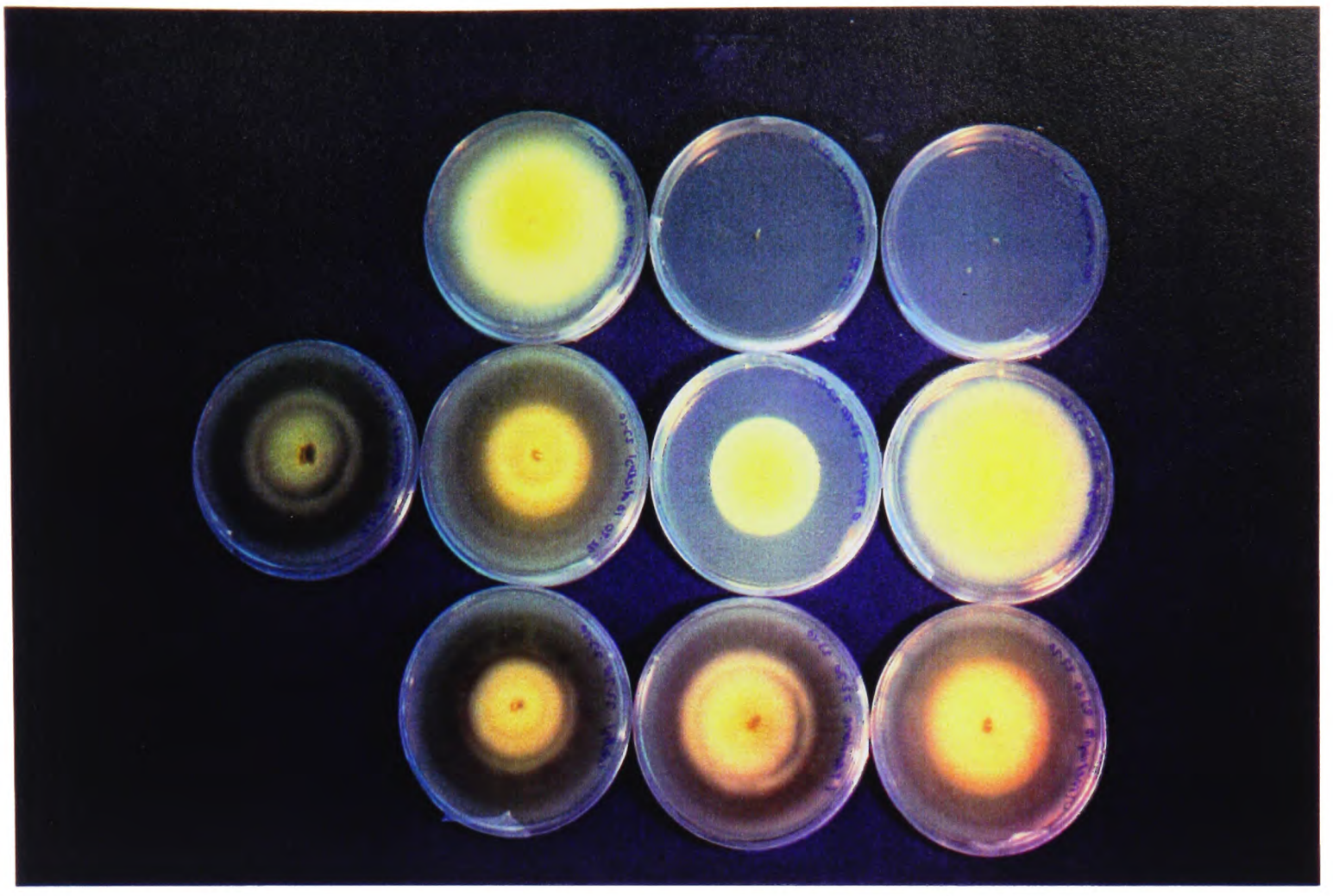
Plate 6.6

Effect of the DOPA melanin-inhibiting compounds and the antibiotic cerulenin after 21 days incubation at 19°C in the dark.

A) Wild-type strain 22-20 grown on MYG containing 1, 10 and 100 μg ml^{-1} tropolone, diethyldithiocarbamic acid and 2-mercaptobenzothiazole.

B) Wild-type strain C87/631/1 and its colour mutants MC87J, MC87A, MC87K and MC87AA grown on MYG containing 1 and 100 μg ml^{-1} cerulenin.

A


µg ml
100
10
1
Control
Tropolone
Diethyldithio-
carbamic acid
2-mercapto-
benzothiazole

B

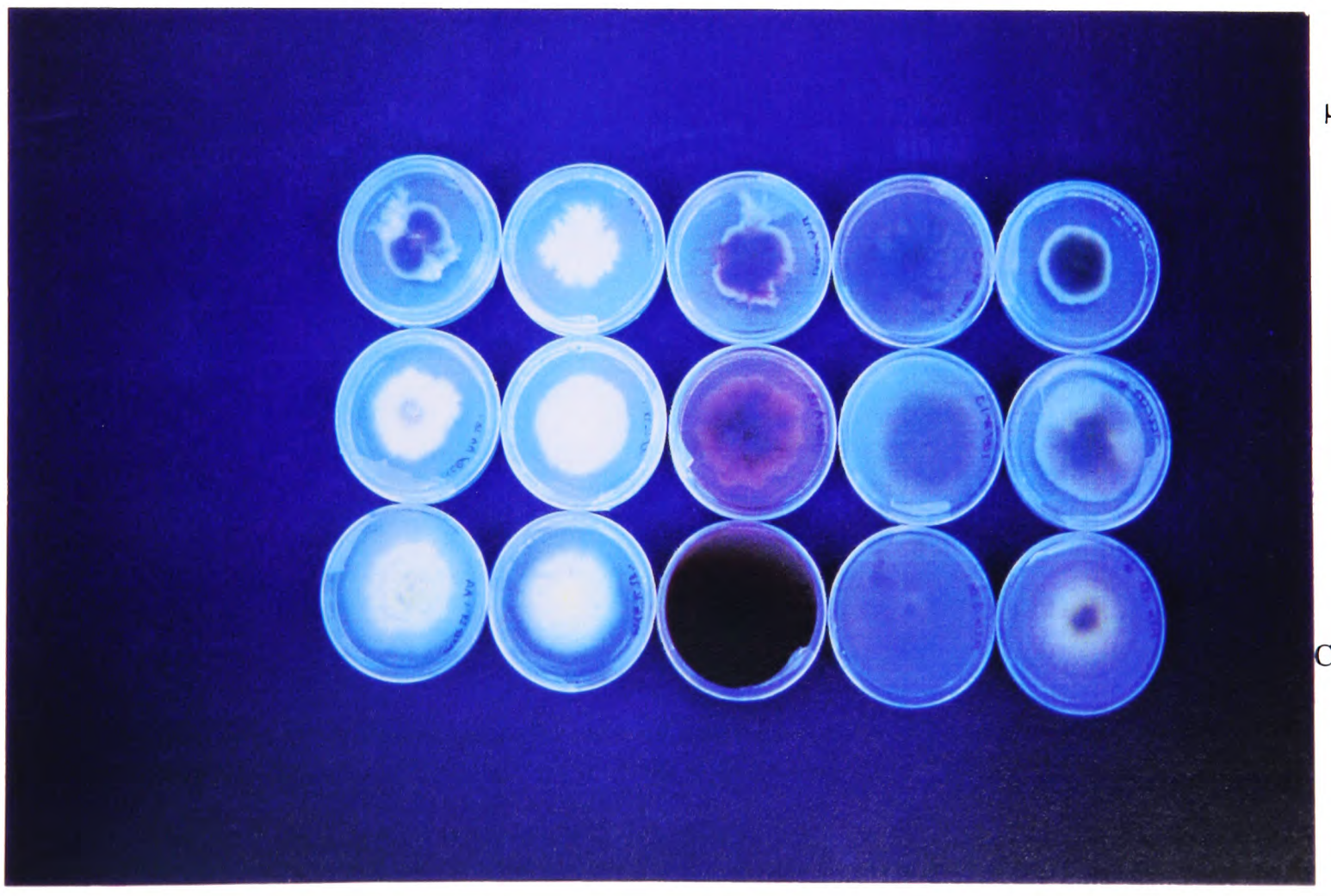

µg ml-1
100
1
Control
MC87AA
MC87k
MC87A
MC87J
C87/631/1

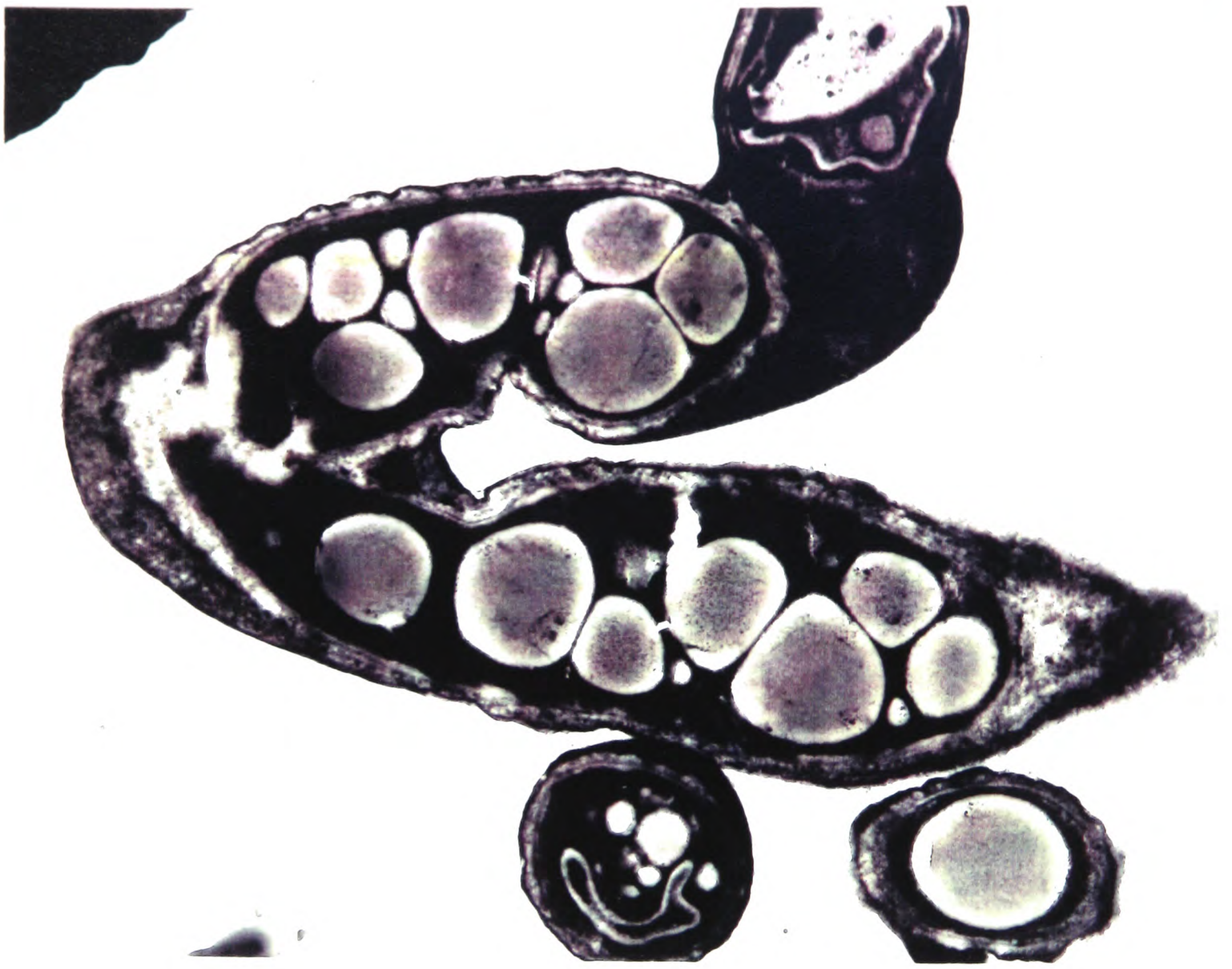
Plate 6.7

Transmission electron micrographs of fungal hyphae from wild-type strain 22-20 and its albino mutant MT183.

A) Wild-type strain 22-20, magnification x 14 000.

B) Albino mutant MT183, magnification x 21 000.

A



B

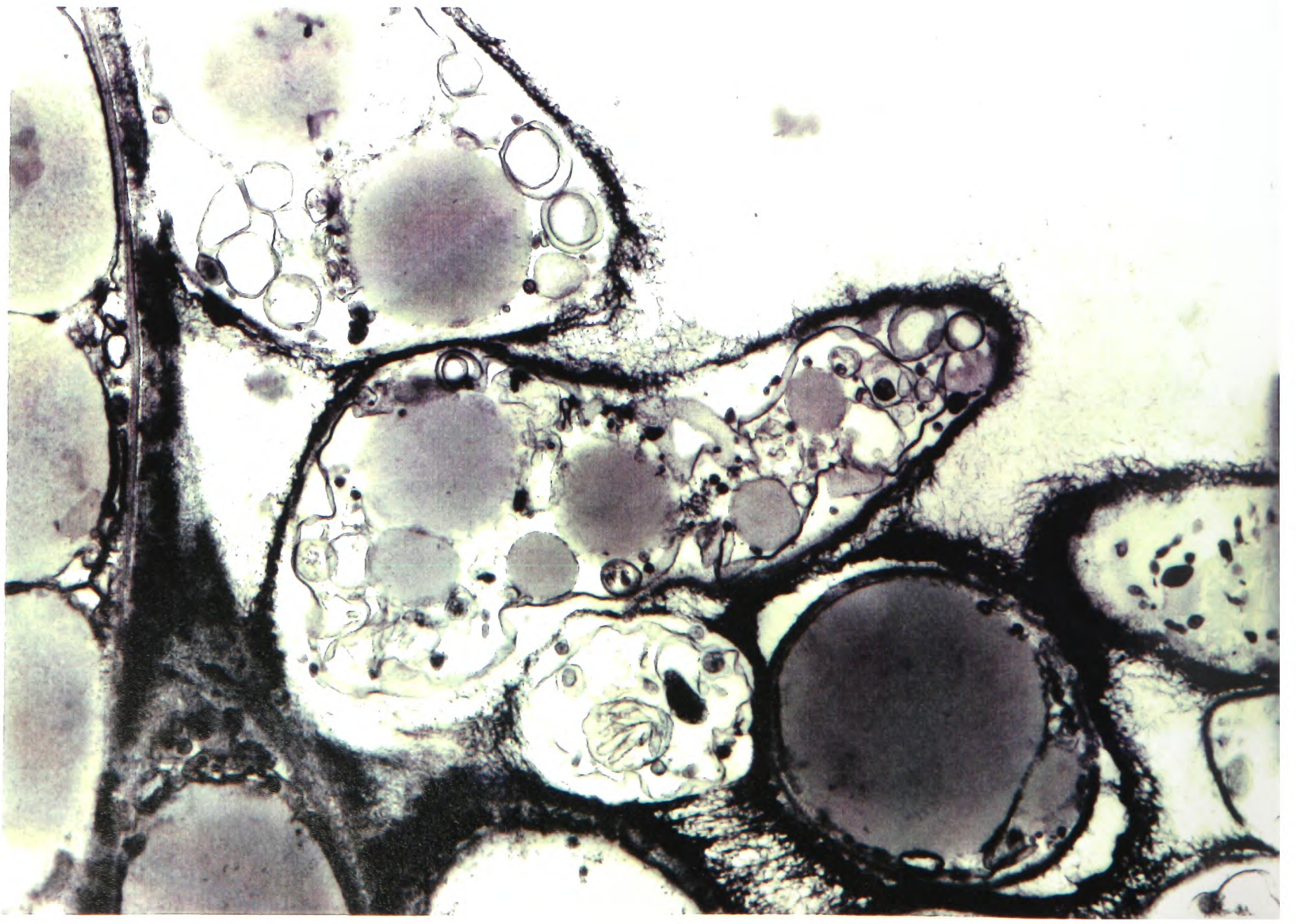


Plate 6.8

Transmission electron micrograph of the wild-type strain 22-20

A) Wild-type strain 22-20 showing granulation (g) in the cytoplasm, magnification x 85 000.

A

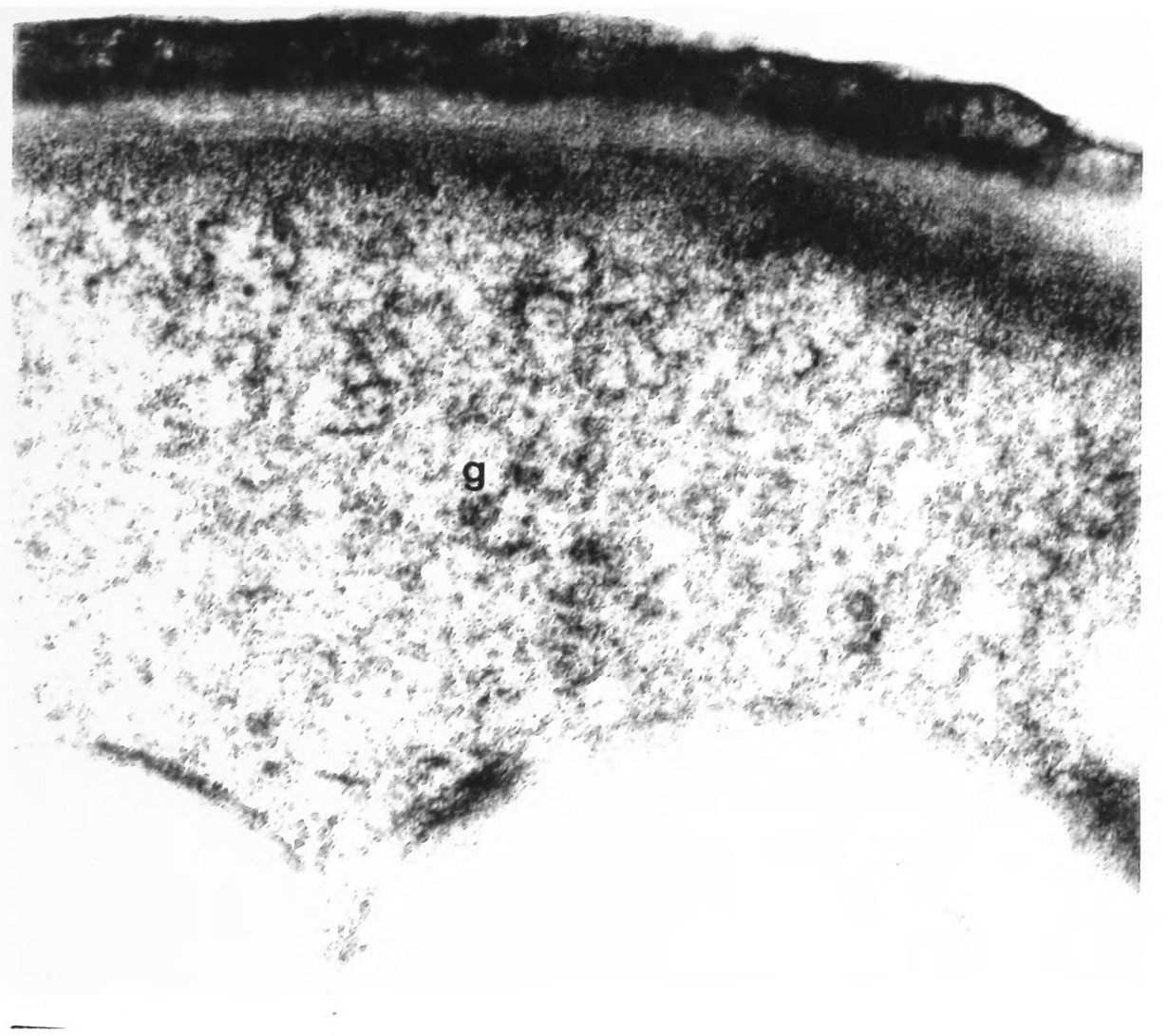


Plate 6.9

Apothecia and ascospore progeny from sexual crosses.

A) Grey/black mature and immature apothecia from a sexual cross between wild-type C87/631/1 and albino mutant MT178.

B) Buff immature apothecia from a sexual cross between buff mutant MC87C and albino mutant MT485.

C) Typical ascospore progeny from a sexual cross between wild-type C87/631/1 and albino mutant MT485.

A

B

C

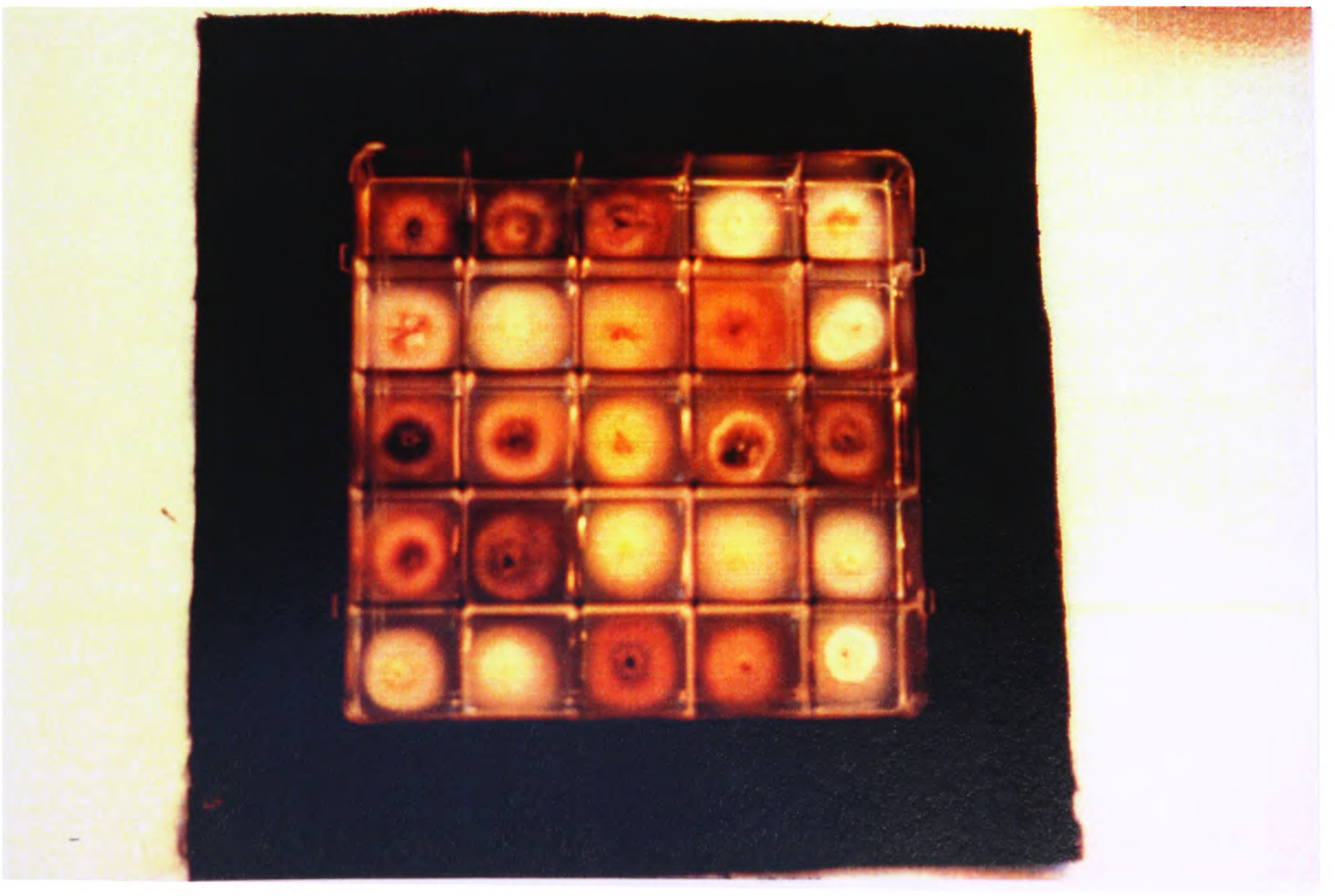


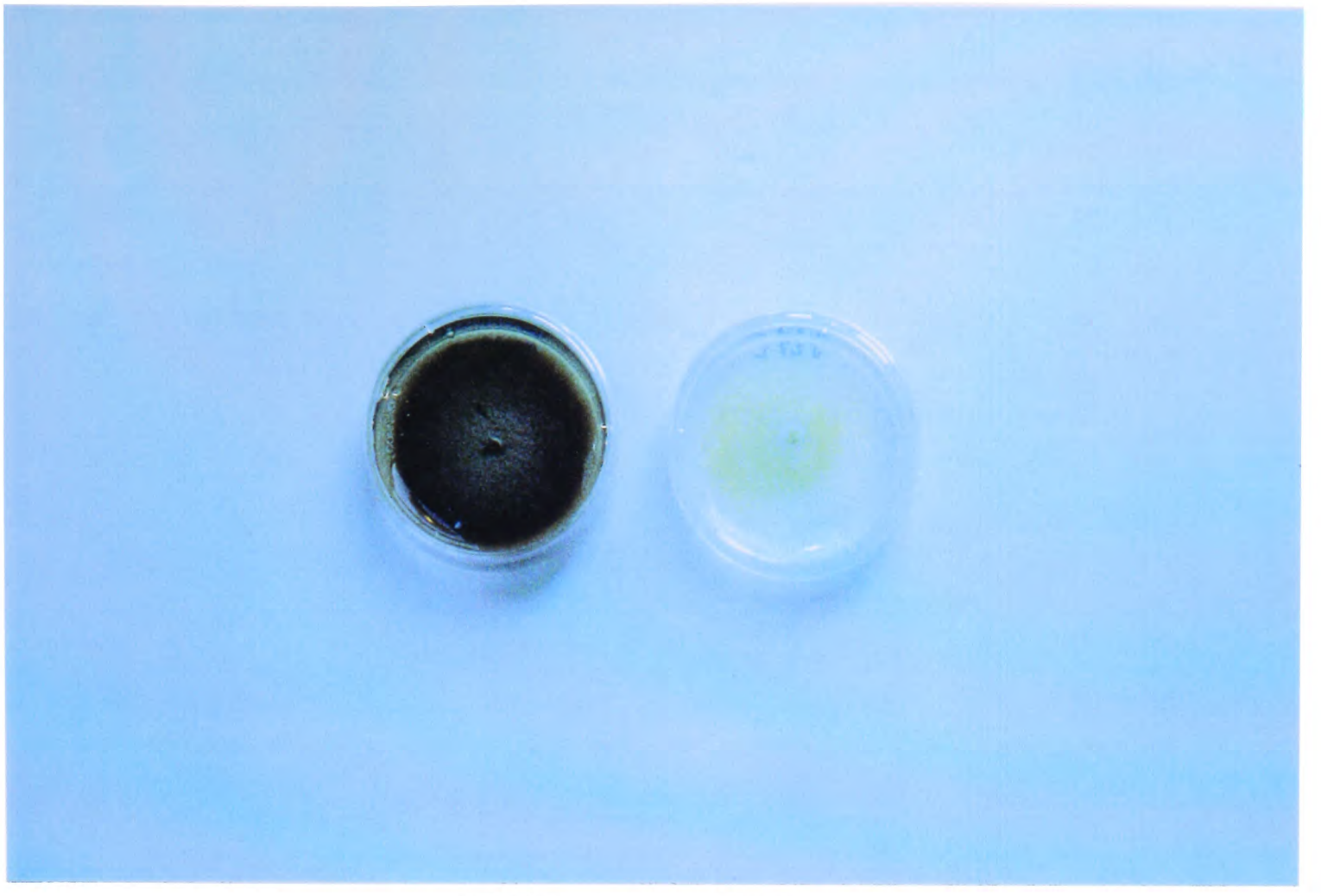
Plate 6.10

The effect of oxidation by sodium hypochlorite.

A) Control plates of colour mutants MC87A (buff) and MT183 (albino) grown on MYG with the addition of SDW.

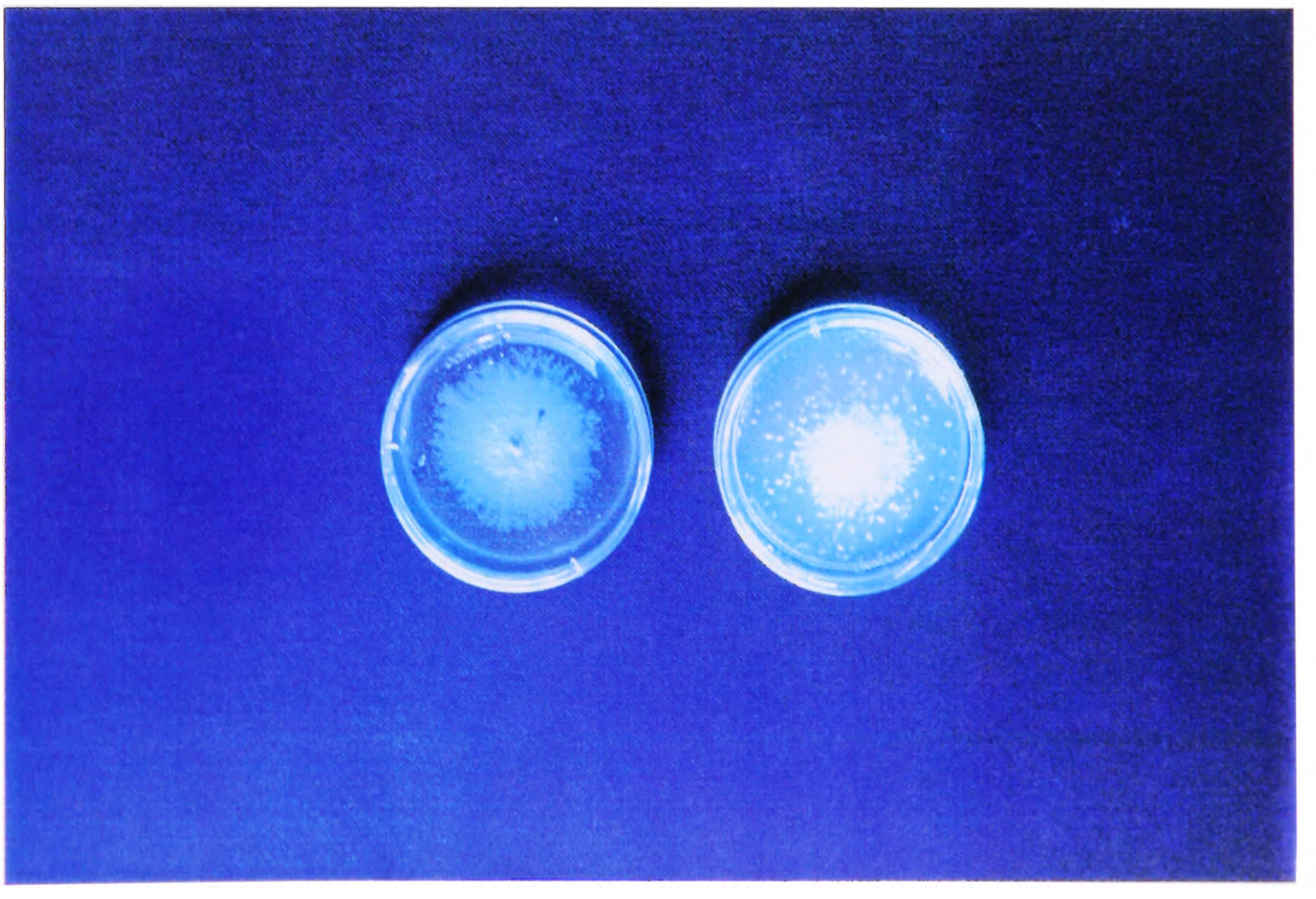
B) Colour mutants MC87A (buff) and MT183 (albino) 80 minutes after the addition of 4% sodium hypochlorite.

A



MC87A MT183

B



MC87A MT183

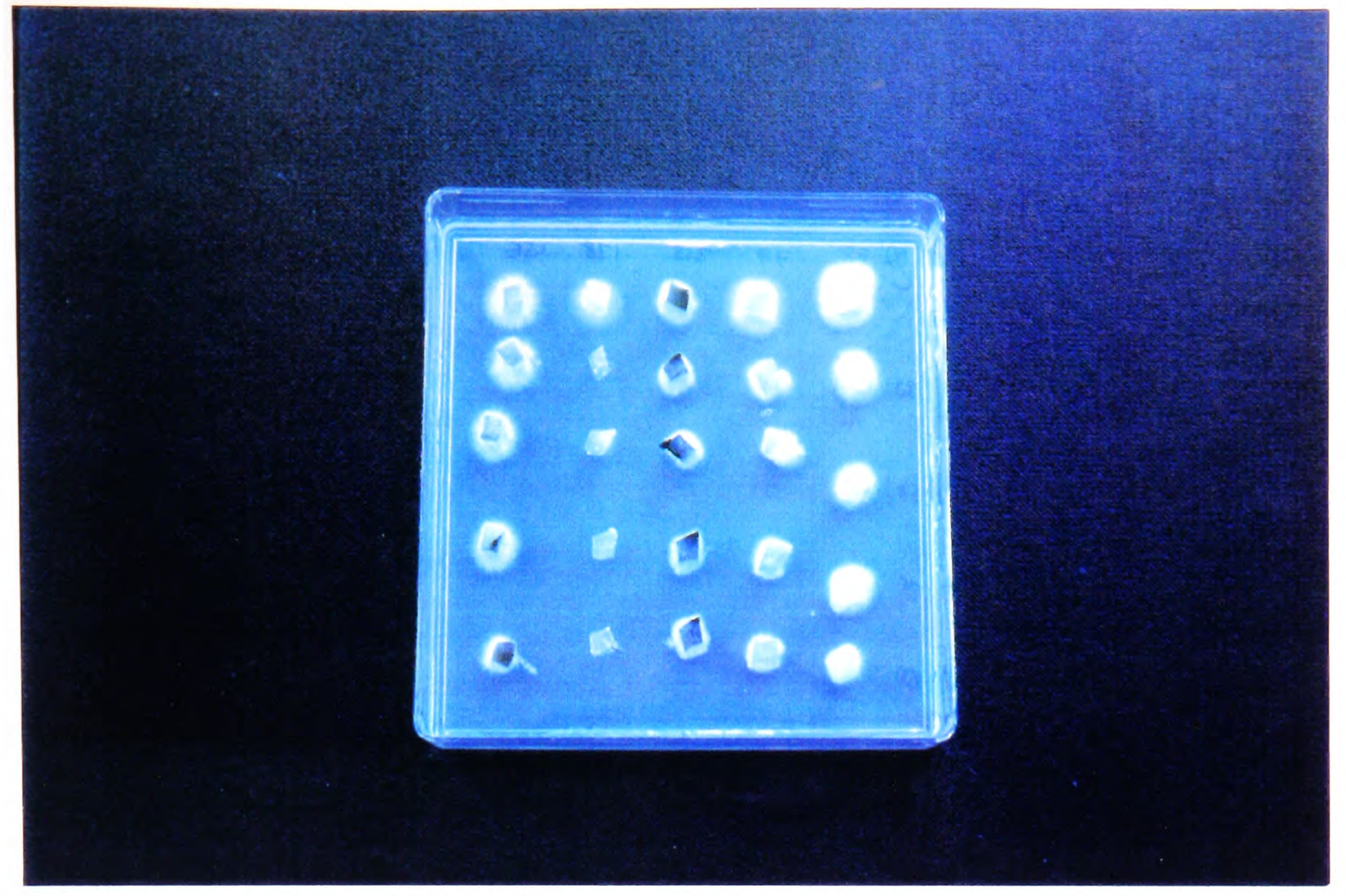
Plate 6.11

The effect on survival of mycelium following UV irradiation and desiccation.

A) Survival of hyphal plugs of colour mutants MT88 (yellow), MT77 (albino), MT215 (brown), MT178 (albino), and MT156 (green) following irradiation for 25, 50, 75 and 100 minutes.

B) Survival of hyphal colonies of wild-type strain 22-20 (black) and colour mutants MT38 (buff), MT77 (albino) and MT178 (albino) grown on cellophane discs following desiccation for 120 and 360 minutes.

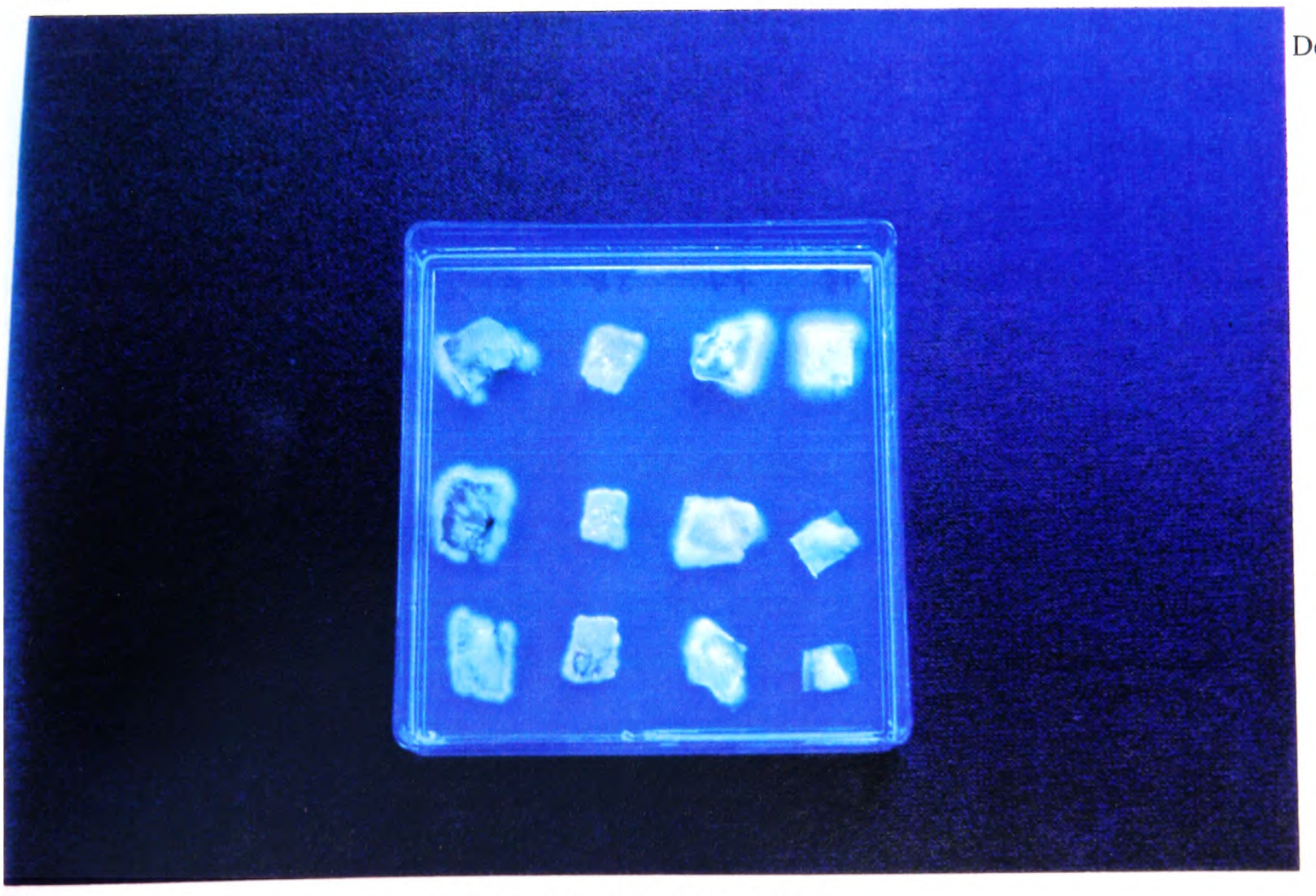
A



UV
(mi
(
2
5
7
10

MT156 MT178 MT215 MT77 MT88

B



Desiccatio
(min)
0
12
36

22-20 MT38 MT77 MT178

6.4 Discussion

A phenotypic range of colour mutants was produced by UV mutagenesis. The colours were found to be similar to those of colour mutants of the ascomycetes *M. grisea* (Chumley and Valent, 1990), *C. miyabeanus* (Kubo *et al.,* 1989), *C. heterostrophus* (Drechsler) Drechsler (Tanaka, Kubo and Tsuda, 1991) and *V. dahliae* (Bell *et al.,* 1976b) which were also obtained from spores by UV mutagenesis or treatment with N-methyl-N′-nitro-N-nitroguanidine and ethyl methanesulphonate. Genetic nomenclature of colour mutants from these pathogens has given rise to the general phenotypic designations of albino (no pigment), rosy (rosy pigmentation), and buff/brm (reddish pigmentation) into which some of the colour mutants from *P. herpotrichoides* could be designated. Additional colour mutations were also isolated and were designated yellow (yellow pigmentation), green (green pigmentation) and brown (brown pigmentation). Petri-dish cultures were found to range in colour intensity. This observation was also found with the colour mutants from *M. grisea* and *C. lagenarium* (Chumley and Valent, 1990; Kubo *et al.,* 1987) and may be attributed to different metabolic rates, perhaps alterations of which may be caused by continuous sub-culturing *in vitro*. Continuous sub-culturing did not reveal any reversions back to the wild-type phenotypes. However when some of the plates were incubated for a considerable length of time (>120 days) two of the albino mutants MT141 and MT77 produced pale grey mycelium at the colony edges. The biosynthesis of pigments in these mutants thus may only be turned on after vegetative growth has almost or completely stopped. Starvation conditions at a maximum stage of growth could induce metabolic development leading to pigmentation. Similar reversions have also been found with albino mutants of *M. grisea* (Chumley and Valent, 1990). Application of the DHN melanin inhibiting compounds fthalide, tricyclazole or pyroquilon to these mutants (MT141 and MT77) would verify that this later produced pigment is melanin if it became a buff colour. Degradation of the compounds over this considerable incubation period (>120days) may however be a problem. The frequency of colour mutants produced from the wild-type strain C87/631/1 was approximately double

that from the wild-type strain 22-20. These frequencies are approximately 10-100 times lower than the frequency of colour mutants production from *M. grisea* using UV mutagenesis (Chumley and Valent, 1990). This may be due to an increased number of cells (3 cells in *M. grisea* and 4-6 cells in *P. herpotrichoides* conidia) and hence more nuclei present in the conidia of *P. herpotrichoides*. It could also be due to different doses of UV irradiation as 312 J m^{-2} at a distance of 6cm was used in the present study compared to 2688 J m^{-2} at a distance of 9cm in the production of mutants from *M. grisea*. No colour mutants appeared spontaneously from the conidia.

The 20 colour mutants used in this study were selected from approximately 180 colour mutants produced. They were chosen for further study on the basis of morphology, growth rates, pigmentation and the ability to produce conidia because the UV irradiation used to produce the colour alterations may have disrupted other vital genes. Of the colour mutants produced about 15% did not sporulate and 6% had an altered phenotype, this being of very slow growth on MYG agar. Selection was made for the accumulation of a large amount of pigment as this gave clear examples of the distinctive phenotypes. Pigmentation was generally more intense in the wild-type strain C87/631/1 and its colour mutants than wild-type strain 22-20 and its colour mutants. The ability to form conidia was checked for any phenotypic alterations. Conidia were also used for pathogenicity testing and in the sexual crosses. Where conidia were produced, germination rates were comparable indicating no obvious physical differences except of colour pigmentation caused by the mutagenesis.

Characterisation of the colour mutants revealed that photo-induction was required for the albino mutants from wild-type strain 22-20 to produce pinky pigments. These were not visible when the albino mutants were grown in the dark. No colour changes in the other colour mutants were seen. However in these latter strains, the pink pigments may have been masked by the other pigments rather than not being present.

Pink pigmentation ascribed to carotenoid synthesis has been shown to be photo-induced in *Wangiella dermatitidis* (Kano) McGinnis (Geis and Szaniszlo, 1984). They found a melanin-deficient strain of *W. dermatitidis* that showed photo-induced carotenoids were more resistant to UV irradiation than the same strain containing no photo-induced carotenoids. Geis and Szaniszlo (1984) suggested that the carotenoid pigments in the wild-type that appeared to be less effective against UV irradiation were being shielded by the superior melanin pigments. They concluded that the carotenoid-mediated protective effect in *W. dermatitidis* was probably involved in the shielding of sensitive molecules or organelles rather than in the neutralisation of harmful oxidants. This may possibly be the role of these pink pigments in *P. herpotrichoides.* A host plant's response to invasion involves peroxidase activity generating a highly oxidising environment. Thus carotenoids may play an important defence mechanism in this environment. There has been no evidence of carotenoids being single pathogenicity determinants in any fungi.

No effect on the intensity or colour of the pigmentation was found by altering the growth media from MYG to PDA. This indicates that the precursors required for pigment production were present in both media.

The wild-type strain C87/631/1 and its colour mutants (except the albinos) secreted pigments of the same colour as the mycelium when grown in shake culture. These are unlikely to be extracellular melanins. Extracellular melanins are synthesised away from the cell walls by either the secretion of phenol oxidases to oxidise phenolic compounds of various origins or by secretion of phenols which are auto-oxidised or oxidised by enzymes later released from the fungus (Bell and Wheeler, 1986). The pigments in the media from wild-type strain C87/631/1 and its colour mutants are probably broken off from fibrils which are an extension of the cell wall. Melanin granules have also been found in association with the fibrillar matrix in *M. grisea* (Bourett and Howard, 1990) and *V. dahliae* (Wheeler *et al.,* 1976). As no pigments were present in the shake culture from wild-type strain 22-20 or any of its colour

mutants this may reflect a reduced intensity of pigmentation in liquid culture or be related to a closer association of the pigments to the cell wall and fibrillar matrix.

Complementation tests showing the presence of heterokaryons were generally unsuccessful, with a general failure to show complementary colour changes. This lack of complementation may have been due to poor diffusion of melanin biosynthesis intermediates or due to no heterokaryon formation. Previous studies examining vegetative compatibility found limited compatibility within W and R wild-type strains but no compatibility was found when auxotrophic mutants were used. (Hocart *et al.,* 1989). The UV mutagenesis may have disrupted compatibility genes. However this is unlikely to have occurred in all the colour mutants. This indicates vegetative incompatibility in the colour mutants produced from *P.* herpotrichoides. Compatibility studies with mutants of *M. grisea* and *V. dahliae* have also proved unsuccessful even though genetic analysis of these mutants indicated that they should complement restoring a wild-type phenotype (Chumley and Valent, 1990; Nagao, Wakatabe and Iijima, 1994).

Three of the buff coloured mutants were found to excrete their reddish pigments into the agar media. Excretion of a reddish pigment has also been seen with colour mutants from *C. miyabeanus* (Ito & Kuribayashi) Drechsler (Kubo *et al.,* 1989) and *V. dahliae* (Bell *et al.,* 1976b) The albino mutants in the present study from both wild-type strains 22-20 and C87/631/1 when grown in combination with the pigment excreting mutants became a reddish colour. This then allows them to be classified as buff mutants. Microscopic studies and the removal of the cellophane based colonies from the coloured medium indicate that the pigment was being taken up by the hyphae of these albino mutants. This reddish pigmentation of the buff mutants (MC87A, MC87D, MC87C, MC87K, MT38, MC87D) is similar to the colour seen when wild-type strains of *V. dahliae*, *C. lagenarium, M. grisea, C. miyabeanus* are grown in the presence of the DHN melanin-inhibiting compound tricyclazole. The colour is similar to the buff mutants produced from these strains, and this reddish

pigment in these other studies has been identified as scytalone (Tokoubalides and Sisler, 1979; Wheeler, 1983; Kubo *et al.,* 1982b; Woloshuk *et al.,* 1981). Buff and brm colour mutants are thought to be inhibited at the same site of action as tricyclazole. This has been shown to be in the conversion of 1,3,6,8-THN → scytalone and 1,3,8-THN → vermelone (Wheeler and Greenblatt, 1988; Bell *et al.,* 1975; Tajima *et al.,* 1989). If scytalone is identified in the media then there is a block at 1,3,8-THN → vermelone. In studies on *C. heterostrophus* and *M. grisea* the mutants are termed salmon and rosy respectively and are blocked at 1,3,6,8-THN → scytalone (Tanaka *et al.,* 1991; Chumely and Valent, 1990). No colour mutants of *P. herpotrichoides* were salmon or rosy but were buff, suggesting a block at 1,3,8-THN → vermelone. This is the same colour seen when the DHN-melanin inhibiting compounds block this step. Mutants of *V. dahliae* termed brm that extruded the pigment identified as scytalone have been found to blacken albino sclerotia. These blackened sclerotia were found to have the same chemical and physical properties as those of the wild-type fungus. (Bell *et al.,* 1976a, 1976b; Wheeler *et al.,* 1976; Wheeler *et al.,* 1978). Genetic analysis of albino mutants from *C. miyabeanus* and *C. heterostrophus* could split them into 2 types by their reaction when co-inoculated with a mutant producing scytalone; one type (type 1) that re-gained a dark green colouration and the other (type 2) that did not. The albino mutants of *P. herpotrichoides* could not be split into different phenotypic groups in the presence of the reddish pigment and they did not gain the phenotypic appearance of the wild-types but that of the buff mutants. This indicates that there may be a genetic blockage further down the biosynthetic pathway in melanin biosynthesis. However this would be unlikely to occur in all the albino mutants tested. The concentration may not have been high enough for conversion to the next stage in the pathway or the buff pigment location in the hyphae may have been in the fibrillar matrix external to the cell wall and thus became inaccessible for further metabolism. Electron micrographs of the development of appressoria of *M. grisea* suggested that melanin biosynthesis is by either a non-vesicular mechanism for the delivery of some precursors and (or) enzymes to sites of melanin synthesis, or alternatively, the melanin layer could be

derived from extracellular precursors and enzymes stored in the periplasm (Bourett and Howard, 1990). This latter possibility is consistent with the observation that albino mutants of *M. grisea* can only form a melanin layer when exogenous scytalone is added (Chida and Sisler, 1987; Wheeler *et al.*, 1976, Wheeler *et al.*, 1978). If the melanin layer was formed by the addition of material from the cytoplasmic side, then a supply of exogenous scytalone would soon be blocked which may be the case for the albino mutants of *P. herpotrichoides.* Studies purifying the reddish pigment would need confirmation that it is scytalone and the addition of this pigment to albino mutants may then give a more accurate picture of the location of melanin biosynthesis.

The three DHN-melanin biosynthesis inhibiting compounds restrained melanin biosynthesis in the wild-types (W and R-types), brown and green colour mutants of *P. herpotrichoides*. The colours changed to reddish brown indicating that melanisation occurs via the pentaketide pathway. The DHN-melanin inhibiting compounds inhibit specifically the reductase enzymes involved in this pathway. The concentration required for optimum inhibition varied for the three fungicides tested. The concentrations though are similar to those seen to inhibit melanin biosynthesis in *Gaeumannomyces* Arx & D. L. Olivier isolates (Elliot, 1995), *V. dahliae* (Tokousbalides and Sisler, 1979) and *M. grisea* (Wolkow, Woloshuk and Sisler, 1980). Melanisation was found to be inhibited without apparent effects on growth, this finding is similar to that of other fungi with tricyclazole being non-toxic to *M. grisea in vitro* at concentrations that control rice blast disease (Froyd *et al.*, 1976; Froyd, Guse and Kushiro, 1978). In the wild-types and colour mutants of *P. herpotrichoides* higher concentrations were found to inhibit melanin biosynthesis completely and reduce growth. The effect on sporulation was not investigated in the present work. However spore formation in *M. grisea* was inhibited in the presence of pyroquilon, thus showing that these fungicides may have an effect on secondary infection (Uehara *et al.*, 1995). Studies on *V. dahliae* found that tricyclazole primarily inhibits the reduction of 1,3,8-THN to vermelone showing enhanced accumulation of

scytalone, and shunt products flaviolin, and 2-hydroxyjugalone (Tokoubalides and Sisler, 1979, Wheeler and Greenblatt, 1988). Tricyclazole has been suggested to compete with the biosynthetic intermediate 1,3,8-THN (Omata *et al.,* 1989; Lundqvist *et al.,* 1993) and also to bind to the reductase enzyme (Vidal-Cros *et al.,* 1994). At higher concentrations in *V. dahliae* tricyclazole has been suggested to inhibit scytalone production seen by the enhanced accumulation of flaviolin. The conversion of 1,3,6,8-THN to scytalone is analogous to the conversion of 1,3,8 -THN to vermelone (see Fig 2.5) (Tokousbalides and Sisler, 1979). Studies on *M. grisea* have cloned and sequenced the enzyme tetrahydroxynaphthalene reductase which catalases the NADPH-dependent reduction of 1,3,6,8-THN into scytalone and 1,3,8-THN into vermelone. This reductase shares 56% amino acid identity with a putative ketoreductase involved in aflatoxin biosynthesis in *A. parasiticus* Speare (Vidal-Cros *et al.,* 1994). The pigments seen at optimum concentration may also be made from 1,3,8-THN or shunt products that accumulate. Other commercially available compounds for the control of rice blast include pyroquilon and fthalide. They have been shown to have similar sites of inhibition as tricyclazole. They are also non-fungitoxic (Woloshuk *et al.,* 1981). These compounds also inhibit pigmentation in some *Aspergillus* and *Penicillium* isolates (Wheeler and Klich, 1995). Therefore the identification of scytalone and shunt products from *P. herpotrichoides* would confirm the same reductase enzyme is involved and that pentaketide metabolites are used to synthesise these pigments. Further studies with *P. herpotrichoides* could also include the application of non-fungitoxic concentrations of tricyclazole found *in vitro* studies to the plants both as curative and preventative measures so as to determine any interactions between the host plant, pathogen and the compounds. Melanin or its intermediates or shunt products caused by auto-oxidation may have toxic effects on fungal or host plant cells leading to reduced virulence of the fungus or greater host plant cell death which may reduce the spread of the fungus or make more nutrients available to it .

The application of cerulenin did not have an inhibitory effect on melanin production in *P. herpotrichoides* unlike the effect seen when cerulenin is applied to *C. lagenarium* where appressorial pigmentation is inhibited. However treatment with scytalone restores appressorial pigmentation in *C. lagenarium* (Kubo *et al.*, 1986; 1987). Cerulenin has been shown to inhibit the malonyl-ACP:acyl-ACP condensation reaction that is found in fatty acid biosynthesis (Giuliano *et al,*1973). It has also been shown to inhibit polyketide synthesis which is considered to be the polymerisation of malonate and acetate via a condensation reaction similar to that of fatty acid synthesis in *C. lagenarium* (Kubo *et al,* 1986). These results indicate that either the condensation reaction of malonyl-ACP:acyl-ACP in *P. herpotrichoides* is not directly involved in melanin biosynthesis or that the concentrations tested were too low to inhibit the reaction. Fatty acid or sterol biosynthesis was inhibited in *P. herpotrichoides*. This was shown by reduced growth and altered colony morphology seen in the presence of high concentrations of cerulenin. The reduced pigmentation intensity seen at the highest cerulenin concentration may have been due to the reduction in vegetative growth.

The yellow and albino colour mutants did not show any change in colour pigmentation with the DHN-melanin inhibiting compounds. This indicates that in these mutants the genetic blocks occur upstream of the 1,3,8-THN / 1,3,6,8-THN steps. No colour change was seen in the buff mutants indicating that a genetic block occurs after 1,3,8-THN. A genetic block downstream of 1,3,6,8-THN cannot however be ruled out as the lower concentration of the DHN-melanin inhibiting compounds are thought to inhibit 1,3,8-THN to vermelone showing the same colouration as the buff mutants. These findings are similar to the studies on the albino mutants of *C. miyabeanus* suggesting that the albino mutants may be defective in pentaketide cyclisation. The buff pigments from *C. miyabeanus* are thought to be derived from a shunt pathway at a deficiency point thought to be after scytalone (Kubo *et al.*, 1989).

As already mentioned the reductase and dehydratase melanin biosynthesis genes have been cloned and sequenced from *M. grisea, C. lagenarium* and *A. alternata.* The 1,3,6,8 THN reductase gene from *M. grisea* is thought to catalyse the NADPH-dependent reduction of 1,3,6,8-THN into Scytalone and 1,3,8-THN into vermelone. This reductase shares 56% amino acid identity with a putative ketoreductase involved in aflatoxin biosynthesis in *A. parasiticus* (Viviani *et al.,* 1993; Wheeler, 1982; Vidal-Cros *et al.*, 1994). The polyketide synthase gene from *C. lagenarium* was able to restore a defective albino mutant back to a wild-type phenotype (Kubo and Furusawa, 1991; Kubo *et al.,* 1991; Takano *et al.,* 1995). Using cDNA probes from *A. alternata* and *M. grisea,* the dehydratase and reductase genes involved in the conversion of scytalone to 1,3,8-THN (Kubo *et al.,* 1996) and 1,3,8-THN to vermelone (Perpetua *et al.,* 1996) have been cloned in *C. lagenarium*. This indicates that the active site residue involved in the common reduction process may be conserved.

To identify genes involved in melanin biosynthesis in *P. herpotrichoides* colour mutants could be transformed with DNA from a wild-type strain genomic library by the construction of cosmids. Alternatively it may be possible to screen a cDNA library of wild-type *P. herpotrichoides* using plaque hybridisation for clones using cDNA probes of melanin biosynthesis genes from *A. alternata, M. grisea* or *C. lagenarium.* Transformation of mutants containing these genes will identify any changes in phenotype especially back to the wild-type and this could be re-confirmed by obtaining transformants by gene disruption.

A yellow pigment was seen concurrently with the growth of the mycelium before darker pigmentation occurred. This yellow pigment may be a stage in the DHN pathway unique to *P. herpotrichoides* or, more likely, it may be a different kind of pigment such as a carotenoid. It could be part of melanin biosynthesis from a different precursor such as catechol. A similar yellow pigment has been observed in culture during melanogenesis in *V. albo-atrum* and from this it was suggested that

catechol was a substrate for melanin in this fungus (Gafoor and Heale, 1971). Most probably this yellow pigment is a carotenoid such as β-carotene. Carotenoids are C_{40} pigments which include open chain compounds such as lycopene. They may be monocyclic such as in γ-carotene or bicyclic such as in β-carotene. Carotenoids are synthesised from geranylgeranyl pyrophosphate units in which the first product phytoene is linked through a *cis* double bond. Stepwise dehydrogenation of phytoene leads to the carotenoids. Carotenoids have been most extensively studied in *N. crassa*. A yellow-orange pigment is produced in conidia and in the mycelium especially in response to blue light. In *Phycomyces blakesleeanus* Burgeff carotenoids are also produced in the mycelium in response to blue light (Munzo, Brody and Butler, 1974; Margalith, 1993). In these species the main contributor to the visible colour is again β-carotene. A late precursor of β-carotene is lycopene, which is red rather than yellow in colour. *N. crassa* conidia normally contain some lycopene as well as much larger quantities of β-carotene. Pigmented mutants *al-1* in *Neurospora* and *car*-A in *Phycomyces* accumulate large amounts of phytoene which is the colourless precursor (Sandmann, 1993; De la Guardia *et al.*, 1971). Purification of this yellow pigment and identification of any phytoene, lycopene or β-carotene directly from the yellow mutants and wild-types of *P. herpotrichoides* would confirm that this material is probably β-carotene, and its synthesis is unrelated to the dark melanin pigments. Earlier blocks such as in the albino mutants in this study cannot account for either carotenoid or melanin synthesis. Testing for phytoene in these albino mutants would be needed to confirm this.

Sexual crosses analysing the colour mutants revealed that the albino mutant phenotypes were probably due to a single unlinked gene defect segregating 1:1 albino mutant to wild-type. The ratios were not always precisely 1:1 which may have been due to the randomly isolated products of meiosis used to obtain their phenotypes. In some cases the sample size may not have been large enough. Progeny from the albino crosses using mutants MT141 and MT77 may have also given slightly inaccurate results because the mutants have been found to produce pigments

much later in growth and thus in the small compartments in which the progeny were grown they may have already started to turn black. This was counted as a brown/black colour instead of an albino. Examination has not been made to see if any tetrad analysis (ordered or unordered) of the products of meiosis can be made from the asci in the apothecia of *P. herpotrichoides.* This analysis would give better evidence of a single gene defect and the sequence of segregation. The crosses made with the yellow mutants with the wild-types did not segregate 1:1 as a number of different coloured progeny were obtained, some of which such as the buff phenotypes may be attributed to spontaneous mutations. Departures from gene segregation of equal frequency can be attributed to linkage which may be the case with the yellow pigment. Further crosses examining this and perhaps also combining mating type of the progeny may reveal any linkage. The possibility of asexual conidia from the parental strains/mutants being present in the ascospore progeny cannot be ruled out, although all possible steps were taken to ensure that just ascospores were present. In this study the buff mutant MT38 was sexually infertile so examination of other buff mutants with wild-types would indicate if a single gene is involved here. The different colony morphologies indicative of sexual progeny have been found in other studies (Nicholson *et al.,* 1991a; Dyer pers comm, 1998). Where the wild-types were used in sexual crosses all apothecia were grey/black. Crosses between albino and buff mutants gave buff apothecia. This indicates an epistastic relation of these genes being black dominant to buff and albino and buff dominant to albino. These are consistent with the order of function being albino→ buff → brown/black in melanin biosynthesis but assumes that pigmentation biosynthesis in the apothecia is via the same pathway in the hyphae and that nuclei from both parents are involved in apothecia formation and thus its pigmentation. Apothecia formation in *P. herpotrichoides* has not been investigated. It is possible that the structure of the apothecia is produced by just one of the parents, probably the most fertile. Thus confirmation of the suggested epistasis relationships would need to be performed by analysis of the progeny of black x buff, buff x buff and buff x albino crosses. The progeny from the brown colour mutant MT215 with the wild-type C87/631/1 were

all brown and this probably indicates that the brown to black colouration depends on the extent of polymerisation/oxidation of the 1,8- DHN units which may be reduced in the brown and even the green colour mutants. The genetic analysis of the yellow mutants should be further investigated but the yellow pigment probably cannot be related to the melanin biosynthetic pathway if it is confirmed a carotenoid. Chumley and Valent (1990) performed genetic crosses of albino, rosy and buff mutants of *M. grisea*. They found that the three mutant phenotypes were due to single gene defects at un-linked loci segregating 1:1 mutant to wild-type phenotypes. Further genetic crosses yielded three possible classes of double mutants showing epistasis relationships; the albino rosy and albino buff mutants being albino and the rosy buff mutants were rosy. They concluded that the epistasis relationships were consistent with the order of function albino → rosy → buff in melanin biosynthesis. These unlinked pigmentation genes coding for enzyme activities (Chumley and Valent, 1990) have been mapped in *M. grisea* (Romao and Hamer, 1992). An albino mutant from *V. dahliae* was found also to be due to a single gene segregating in a 1:1 mutant to wild-type ratio when analysed via the parasexual cycle (Bell *et al.*, 1976b). Sexual crosses of the progeny of varying phenotypes would further characterise the genetic basis of melanin biosynthesis in *P. herpotrichoides.*

Ultrastructural investigations were made to locate the site of melanin deposition in the hyphae of *P. herpotrichoides.* The transmission electron micrographs produced did not reveal a definite location of the melanin granules due to the heavy staining of the cytoplasm. Staining for less time and with less lead citrate did not improve the contrast of the granules relative to the cytoplasm. The alternative fixative of $KMnO_4$ has been shown to make melanin granules more apparent and may improve the identification of the granules in the wild-type (Wheeler *et al.*, 1976, 1978). The addition of catechol or DOPA to albino mutants from *V. dahliae* enabled them to form melanins. However the deposition of the melanin granules was indiscrete and uniformly distributed along the extracellular fibrils and in the outer microsclerotial walls. These melanins could only be demonstrated with $KMnO_4$ fixation whereas

wild-type melanin or melanins formed by the mutants with scytalone or DHN were apparent after fixation with OsO_4 or $KMnO_4$ (Wheeler *et al.,* 1978). Apart from the absence of pigmentation the albino mutants produced from *P. herpotrichoides* appeared to have a lack of structure to the cytoplasm. This may have occurred because other genes could have been disrupted by the UV mutagenesis. Other colour mutants thus should be examined by transmission electron microscopy and compared to the wild-type phenotype. These may have fewer structural differences and thus resemble more the wild-types except in pigmentation. Electron micrographs have previously determined that in most hyphae, conidia and sclerotia walls of melanised fungi there are two distinct layers, an inner layer that is electron translucent and an outer layer that may be covered by electron dense granules. Many walls also have a fibrillar matrix that extends outward from the cell wall in which melanin granules are often scattered. It was seen that in *P. herpotrichoides* melanin granules were found to be located in the cell wall. This is in contrast to the pathogens *M. grisea* and *C. lagenarium* whose appressoria have a homogenous melanin layer about 100nm thick positioned just outside the plasma membrane (the location of melanin in the vegetative hyphae of these fungi has not been studied) (Howard and Ferraria, 1989; Bourett and Howard, 1989; Kubo *et al.,* 1985; Kubo and Furusawa, 1986). In the appressoria, this melanin layer is not observed over the pore wall and is thought to maintain a high solute concentration needed to create a high internal osmotic pressure. The non- melanised pore area of the appressoria is probably the only area through which molecules larger than water can pass from the fungus to the underlying substrate. A comparable layer was not present in albino, buff or wild-types treated with tricyclazole of *M. grisea* and *C. lagenarium* except upon the addition of scytalone which restored wild-type appressorial melanisation to these albino mutants (Howard and Ferraria, 1989; Kubo *et al.,* 1985). In contrast melanin in *V. dahliae* appears as granules in the cell walls and fibrillar network. The addition of scytalone to albino mutants allowed the formation of melanin granules similar in appearance and distribution to that of the wild-type (Wheeler *et al.,* 1976; Wheeler *et al.,* 1978; Ellis and Griffiths, 1974). The addition of tricyclazole eliminated or greatly

diminished the appearance of electron dense granules in chylamadospores walls of *T. basicola* (Howard and Valent, 1996). The location of melanin granules in *P. herpotrichoides* is similar to that of DHN melanised pathogenic fungi that do not require melanin for pathogenicity but require it for a role in protection from environmental extremes. Further electron micrographs with the buff mutants, albino treated with scytalone and wild-types treated with tricyclazole would go further in confirming that the granules in *P. herpotrichoides* are DHN melanin.

The pathogenicity on wheat of the colour mutants found that the yellow, green and brown mutants were of low or zero pathogenicity, with the albino and buff mutants being of higher pathogenicity. The DHN melanin in *P. herpotrichoides* thus does not appear to play a direct role in pathogenicity. It has also been found that DHN melanin does not appear to function in pathogenicity of *C. miyabeanus* (Kubo *et al.,* 1989), *V. dahliae* (Bell and Wheeler, 1986), *A. alternata* (Tanabe *et al.,* 1995) and *Gaeumannomyces* isolates (Elliot, 1995). In all cases pigment deficient mutants were fully pathogenic. The zero or low pathogenicity in some of the colour mutants of *P. herpotrichoides* could be explained by other mutations caused by the UV mutagenesis. The role of DHN melanin in *V. dahliae* is believed to be in long term survival of propagative structures such as microsclerotia (Bell *et al.,* 1976b). Examination of pathogenicity of *Gaeumannomyces* isolates referred to the hyphae associated with infection, and determined them as hyaline. However melanisation was seen to occur in the runner hyphae (Elliot, 1995). The melanised hyphae are thus more likely to be associated with survival rather than pathogenicity. In this study an examination was made of infection structures after 8 weeks. However it was not possible to determine what structures were melanised because of the fluorochrome staining used. The darker cells of the infection plaques probably hadn't taken the stain because either an insufficient length of time was left before examination or other structural features were present. Studies on infection plaques on wheat by W-type strains found that they became melanised. An alternative method of clearing and staining of the leaf sheath was used in this study consisting of lactic acid and trypan

blue (Daniels *et al.,* 1995). The next step in determining the effect of melanin on pathogenicity would be to determine what structures became melanised and when this occurred after initial inoculation, perhaps using this alternative staining method. This experiment was only performed on wheat. Thus an extension on to another host such as barley would be valuable. The lack of pathogenicity of some of the green, brown and yellow mutants may be due to other genetic blocks in determinants of pathogenicity such as enzymes involved in plant cell wall degradation or in adhesion of the spores to the coleoptile that may have occurred during UV mutagenesis. In contrast to the above findings DHN melanin has been found to play a direct role in pathogenicity of *C. lagenarium* and *M. grisea.* Colour mutations and application of tricyclazole inhibit appressorial pigmentation in *C. lagenarium* and *M. grisea.* The colourless appressoria germinate laterally and were not able to penetrate nitrocellulose membranes or host leaves (Kubo *et al.,* 1982a, 1982b, 1987; Howard and Ferrari, 1989; Chumley and Valent, 1990). Addition of scytalone restored pathogenicity and darkened mutants of albino but not rosy or buff mutants of *M. grisea* and *C. lagenarium* (Chumley and Valent, 1990; Kubo *et al.,* 1986, 1987). In these fungi other factors apart from melanin have been shown to play a role in pathogenicity. Mutants of *C. lagenarium* identical in shape and colour to the parental strain produced appressoria defective in morphogenesis of the penetration peg and in cellulose degrading enzymes. These mutants were unable to penetrate nitrocellulose membranes or cell walls of the host plant unless transformed using a gene from the wild-type genomic library thus revealing additional essential processes for penetration by appressoria (Katoch *et al.,* 1988; Perpetua *et al.,* 1994). Application of scytalone to the conidia of the albino mutants of *P. herpotrichoides* should not alter their pathogenicity and this would additionally confirm that DHN melanin plays a different role in this fungus.

Analysis of the early infection pattern of both W and R-types of *P. herpotrichoides* on wheat seedlings indicated that there was no evidence of melanisation occurring in conidia, appressorial formation or in runner hyphae (Daniels *et al.*, 1991; 1995). This

may have been because melanisation occurs by secondary metabolism and only the early stages of infection were studied. This may not have allowed sufficient time for melanisation of the runner hyphae to take place. Both cellulolytic and pectolytic enzymes are known to be secreted by *P. herpotrichoides* and be involved in cell wall softening (Cooper *et al.,* 1988; Hanssler, 1973; Hanssler *et al.,* 1971). They may be pathogenicity determinants.

Although it was not apparent from the 8 week assessment that albino strains were less aggressive than the pigmented and darker pigmented colour mutants. This may have been the case earlier on in infection. A time course of study might reveal any infective differences and abilities to survive host cell responses to infection. Infections of laboratory animals with melanin-deficient mutants of *Cryptococcus neoformans* and *W. dermatitidis* (a dematiaceous fungus which grows *in vitro* primarily as a yeast) are not as aggressive as infections by melanised strains (Dixon, Polak and Szaniszlo, 1987; Dixon, Szaniszlo and Polak, 1991). An electron spin resonance study of the pigmented cells of *W. dermatitidis* revealed a stable free-radical population which was lacking in melanin deficient cells making them more susceptible to killing by nitrogen- and oxygen-derived radicals (Wang, Aisen and Casadevall, 1995).

A possible role of melanin in *P. herpotrichoides* infection may be in maintaining turgor pressure in the hyphal cells. It seems likely that fungal turgor pressure is an obligatory requirement for penetration and ramification through plant tissues. Alternatively melanin may combine with plant host enzymes such as chitinase or glucanase used in defence. Observations by Daniels *et al,* (1991) of infection plaque cells of *P. herpotrichoides* revealed that during cell inflation lateral expansion was restricted resulting in growth extension at right angles to the leaf sheath to form elongated cells. This, in combination with extracellular mucilage secreted around the cell tip, was suggested to provide support against which turgor or mechanical pressure could be generated for cell wall penetration (Daniels *et al.,* 1991). Several

functions have been proposed for appressorial melanin:1) to limit the vertical direction of the penetration peg with the pore wall lacking melanin (lateral or no peg emergence is found with melanin less appressoria of *M. grisea* (Howard and Ferraria, 1989) or *C. lagenarium* appressoria (Kubo *et al.*, 1982a; Kubo, 1986)), 2) to make the appressorial wall rigid (Kubo *et al.*, 1982a; Wolkow *et al.*,1983) and 3) to generate internal hydrostatic pressure by maintaining a high internal solute concentration in the appressoria, the discrete melanin layer acting as a barrier to solute efflux. (Howard and Ferrari, 1989). It is well documented that expansion of the hyphal cell wall at the growing apex is forced by turgor (Money, 1995). Measurements of turgor involved in penetration have compared intracellular ice and ice in the fluid surrounding appressoria of *M. grisea* formed on plastic coverslips and non-biodegradable mylar membranes varying in hardness (to eliminate the activity of extracellular enzymes). Much higher turgor pressures were found in wild-types compared to melanin-deficient appressoria (Money and Howard, 1996, Howard *et al.*, 1991). Albino deficient mutants of *M. grisea* were plasmolysed by low solute concentrations compared to melanised appressoria (Howard and Ferrari, 1989). Measurements of turgor pressure from hyphae and infection plaques produced by wild-types and colour mutants of *P. herpotrichoides* may be able to relate melanin to its role in increasing turgor in infection plaque cells or in hyphal survival.

Tests on the rate of oxidation by bleaching with sodium hypochlorite revealed that the presence of pigments increased the length of time taken to be oxidised. The results indicate that the pigments are able to lose electrons and it was seen that the greater intensity and darker the pigment the slower the rate of oxidation. Melanin polymers are formed by the oxidative polymerisation of DHN; consequently it is one of the few known stable free radicals and it can be a 'sponge' for other free radicals. The unique structure of melanins allows them to act as either proton donors or receivers. Thus oxidation by sodium hypochlorite is commonly used to indicate that a compound is melanin. The effect of bleaching by oxidising agents indicated the identity of the dark material found in hyphae of *R. solani* as melanin (Potgieter and

Alexander, 1966). These results indicate that the black, brown, green and buff pigmentation have a greater ability to absorb more free radicals than the yellow and albino mutants. The intensity of the pigmentation reflects the amount of free radical absorption. A negative correlation was found between the length of time needed to bleach sclerotia of *B. cinerea* and the tricyclazole concentration in the medium on which they were produced (Zeun and Buchenauer, 1985). The antioxidant function of melanin has been demonstrated with the melanolytic fungus *C. neoformans* which causes infection of the central nervous system in mice . Addition of oxidants including sodium hypochlorite afforded increased growth in wild-type compared to a melanin deficient strain. The antioxidant capacity conferred by melanin was found to equal that of oxidant production of stimulated macrophages used in defence (Jacobson and Tinnell, 1993). Titration of DHN melanin *in vivo* in *W. dermatitidis* and *A. alternata* with various oxidants including permanganate and hypochlorite neutralised more oxidant than corresponding albino strains (Jacobson, Hove and Emery, 1995). Further evidence of the antioxidant function of melanin is found by monitoring the levels of superoxide dismutase (SOD). An inverse relationship has been demonstrated with the superoxide dismutase in *C. neoformans.* An increase in SOD was seen with albino mutants and a decrease in the level of SOD was found with wild-type strains when they reached the stationary growth phase and melanin was being synthesised (Jacobson, Jenkins and Todd, 1994).

Exposure to UV irradiation decreased the ability of hyphae of *P. herpotrichoides* to re-grow. Hyphal re-growth after UV irradiation was found to be dependant on the presence and intensity of pigmentation. A decrease in lag phase and rate of re-growth of the wild-types and pigmented colour mutants compared with the albino mutants indicated that the pigments present absorbed the free radicals generated by the irradiation. There was probably less damage to the nucleic acids and cell membranes, allowing the cells to survive for longer. A lack of melanin ensured a greater cell destruction and hence an increased lag phase before any re-growth was seen. A high proportion of melanin producing micro-organisms have been associated with

environmentally stressed areas such as the desert, the alpine regions and the upper biosphere. Fungi with melanised spores are more resistant to killing by UV light or solar radiation than ones with hyaline spores and the degree of protection has been found the be proportional to the melanin concentration in the spore walls (Durrell, 1964; Bell and Wheeler, 1986). Similarly it has been found that radioresistance of melanised spores to γ-radiation and x-rays is greater than that of hyaline spores (Zhdanova, Gavryushina and Vasilevskaya, 1973a). A mutant of *Bacillus thuringiensis* Berliner producing a dark pigment showed increased UV resistance and insecticidal activity (Patel *et al.,* 1996). It has been suggested that melanins absorb various types of radiation and dissipate energy primarily by undergoing reversible changes in free radicals (Bell and Wheeler, 1986). Consequently the cytoplasmic membrane in melanised cells is spared from the damaging effects of free radicals formed by irradiation.

The effect of desiccation showed similar results to that of UV irradiation with the presence of pigment compared to albino mutants allowing for better survival after desiccation. It has already been mentioned that melanin may maintain a high solute concentration in the hyphal cells and this would aid retention of a high osmotic potential thus keeping water within the cells compared to a low solute concentration and water leaving the cells. Microsclerotia from albino and dehydratase deficient mutants of *V. dahliae* were unable to survive desiccation in the soil whereas wild-type and albino microsclerotia melanised by treatment with scytalone became dormant when desiccated (Zhdanova *et al.,* 1973b).

Isolation of infection plaques and runner hyphae from host tissue and the effect of UV radiation, desiccation and oxidation on these infection structures may provide direct evidence of the role of melanin in *P. herpotrichoides* being able to survive on host plants. Further studies by sexual crosses using the colour mutants (that have been generated from opposite mating types) and wild-type strains may produce apothecia that are albino. Studies of these apothecia in relation to UV radiation,

desiccation and oxidation may show another related role of melanin in these long term survival propagative structures.

Melanins in fungi also appear to be important for resistance to microbial attack and accordingly most of the fungal biomass in soils is melanised. The resistance of cell walls of *A. nidulans* to microbial lysis has been directly correlated with the melanin content of the mycelium (Kuo and Alexander, 1967; Polacheck and Rosenberger, 1977). Successful survival in the soil has been associated with dark pigments in the cells wall. A comparison of hyphal walls of *Sclerotium rolfsii* Sacc. with the dark rind cells of sclerotia showed them to be less resistant to enzyme hydrolysis by streptomycete culture filtrates, chitinase and β-(1,3) glucanase. (Bloomfield and Alexander, 1967). An increased resistance to microbial lysis of cell walls of mature cleistothecia of *Sphaerotheca mors-uvae* (Schivien.) Berk & Curtis could also be related to their higher melanin content (Jackson and Gay, 1976). Apart from spore survival Lockwood (1960) found that dark hyphae produced by *H. sativum* Pam, King & Bakke, *R. solani* Elle. & Mart. and *A. solani* were resistant to degradation in natural ecosystems. It is implicated that important survival organs of plant pathogenesis are resistant sexual or asexual spores rather than vegetative mycelium. *R. solani* does not form spores profusely and the presence in the walls of a melanin like material has been found to protect the hyphae from microbial lysis when comparing with hyaline filaments (Potgieter and Alexander, 1966). Two anti-fungal compounds (one an antibiotic) have been detected in culture filtrates from *P. fluorescens* (Trevisan) Migula which are inhibitory to spore germination and hyphal growth of *P. herpotrichoides* (Clarkson and Lucas, 1997). Further experiments could use these anti-fungal compounds on the vegetative hyphae and apothecia from wild-types and colour mutants of *P. herpotrichoides,* and relate the rate of inhibition to the presence of pigments. In addition to this, the fungal hyphae could be placed on non-sterile soil and microscopic studies could then determine cell wall degradation caused by microbial lysis. The use of hyphal plugs placed on agar containing micro-organisms that are antagonistic and the measurement of the zones of inhibition of

radial growth or clear zones devoid of bacteria could indicate this possible protective role of melanin in *P. herpotrichoides*. Many hypotheses have been suggested as to how melanin allows protection from microbial lysis: these include shielding from biodegradable polysaccharides of fungal cells walls by overlaying or combining with them or the melanin itself may inhibit one or more of the enzymes participating in lysis of the fungi (Kuo and Alexander, 1967). The protective agent itself must be long-lived or re-generated as rapidly as it is destroyed which melanin is. In the appressorium, melanin may inhibit lateral germination through suppression of appressorium derived wall-splitting enzymes (Kubo and Furusawa, 1991).

These results indicate that the DHN melanin found in *P. herpotrichoides* is associated with the cell walls. It is not directly involved in pathogenicity but in protection from environmental extremes. DHN-melanin inhibiting compounds may be useful to decrease melanisation of hyphae making them more susceptible to environmental extremes or microbial lysis. Alternatively they may allow the build up of toxic intermediates or shunt products.

CHAPTER 7

GENERAL DISCUSSION AND FUTURE RESEARCH

This general discussion summarises and relates the major findings from the results chapters and identifies possible areas of future research. The aim of this study was to expand on the biology of the main W and R pathotypes of this fungus and their relationships with the cereal hosts, and to examine mechanisms which may be involved in pathogenicity. This work used field isolated wild-type strains, parasexual recombinant hybrids produced from crosses between W and R-types and an R-type with *P. anguioides,* and also involved the production of colour mutants from W-types for the study of melanin in *P. herpotrichoides*. Limitations of the time available and resources has lead to some of the areas of work not being covered as extensively as desired. However the initial aims of the work have been satisfied. In general the molecular techniques gave rapid reliable identification of the W and R-types compared to the morphological and pathogenicity identification techniques which were less reliable and more time consuming. Molecular characterisation confirmed the genetic inheritance from both parental strains in the parasexual hybrids. Some of W x R hybrids showed an intermediate morphology and others showed novel pathogenicity. The W and R-types were found to have different infection strategies which can be applied to the traditional pathogenicity characterisation of these pathotypes on wheat and rye and also supports divergence of these pathotypes into the separate species of *T. yallundae* and *T. acuformis* respectively. The ability to produce infection plaques *in vitro* and *in vivo* appears to be an important factor in host colonisation with the numbers produced by the strains and hybrids related to their pathogenicity on wheat. No secondary metabolites were detected from culture filtrates and thus from this study they are not thought to play a role in pathogenicity or symptom induction. The role of melanin in this fungus was found to be protection from the environment and the pigment is synthesised via the DHN melanin-biosynthetic pathway common in other ascomycetes. A possible single gene is

suggested to be causing the albino phenotype but the other colour phenotypes appear to have a more complicated genetic basis.

It is important to identify accurately the pathotype of wild-type strains, as the W and R -types have been shown to have different epidemiologies in the field. The R-type has been seen to cause infections later in the season compared to the W-type (Goulds and Fitt, 1990). This is important in relation to disease severity as if only the W-type is detected then severe late epidemics probably will not occur, but if the R-type is present then late epidemics are more likely and thus early control might be advised. Fungicide resistance has occurred on winter wheat and barley to the MBC fungicide carbendazim (Yarham, 1986; Hollins and Scott, 1987) and the use of the DMI fungicides used to control MBC resistant strains tends to select in favour of the R-type (King and Griffin, 1985). Thus correct pathotype identification can help manage what fungicides or combination of fungicides should be used in disease control and help prevent more fungicide resistance occurring. Identification of the correct pathotype can also help in the understanding of the occurrence of sexual reproduction in the field which may lead to the faster development of fungicide resistance. Current work indicates that the W-type produced apothecia more readily in the field than the R-type (Dyer *et al.,* 1993a). Application of a DMI fungicide would therefore decrease sexual reproduction selecting in favour of the R-type. The initial work of this study used morphological, pathogenicity and molecular techniques to characterise wild-type strains and recombinant hybrids. The cultural and pathogenicity techniques varied in their assignment of the W or R-types to the field isolated strains and this has been a common problem in the past (Hollins *et al.,* 1985; Julian *et al.,* 1994; Gallimore *et al.,* 1987). When performing pathogenicity tests other factors such as temperature, host plant cultivar and viability of inoculum have been found to contribute to the pathogenicity of strains, and highlight the areas of unreliability of this method showing how important it is to standardise pathogenicity trials (Brown *et al.,* 1984; Hollins *et al.,* 1985; Sanders *et al.,* 1986; Scott *et al.,* 1976; Mauler and Fehrmann, 1987b; Fitt *et al.,* 1987). The molecular techniques

used in this study of the restriction enzyme digests and specific primers identifying the W and R-pathotypes were more reliable and quicker than the cultural and pathogenicity identification techniques. Specific primers are now most commonly used for pathotype identification as they can be used directly on field samples of infected stems, thereby eliminating the need to isolate the fungus first (Nicholson *et al.*, 1997). By using the technique of cultural morphology to classify the recombinant hybrids it was found that they could be classified as having either a W or R-morphology. However some showed an intermediate morphology. The molecular characterisation of these hybrids indicated that the R-type parental genome appeared to predominate in the hybrids and it was found that they produced characteristic R-type infection plaques. Novel pathotypes were seen with some recombinant hybrids appearing to infect rye but not wheat, and some strains appeared to be asymptomatic, infecting the plant but not causing disease symptoms. On closer examination however, the hybrids classed as having novel pathotypes or as being asymptomatic on one host species were of low or zero pathogenicity to the other hosts which suggests a generally low level of virulence in these hybrids. The pathogenicity of selected strains and hybrids was repeated when the comparisons were made between disease symptoms, fungal progress and the quantity of fungal DNA in the host stem base. The infection conditions between the glasshouse and controlled environment pathogenicity experiments differed. The glasshouse had a mean day temperature of 17.1°C and night temperature of 2.7°C and the controlled environment cabinet had a day temperature of 12°C and night temperature of 6°C. The pathogenicity of the same strains and hybrids differed between these experiments with the W-type strains being more pathogenic to wheat and the R-type strains more pathogenic to wheat and rye in the controlled environment experiment compared to the glass house experiment. The W x R hybrids though were always of low pathogenicity but symptoms varied between the host species and two sets of conditions.

The ability to produce infection plaques *in vitro* on the base of glass Petri-dishes by the wild-types and hybrids was found to correlate to their pathogenicity when it was

assessed by the depth of penetration of the disease symptoms on wheat. The role of infection plaques is suggested to be in leaf sheath penetration (Daniels *et al.,* 1991). The current work correlates the presence of infection plaques with successful host colonisation. Thus they may be suggested to be important structures in pathogenicity. Further studies examining the ability of strains and hybrids to produce infection plaques *in vivo* or by producing mutants unable to produce infection plaques would confirm the requirement of infection plaques for successful host colonisation.

Two assays were developed to detect secondary metabolites, a wheat cell suspension culture viability test and a wheat root growth inhibition assay. Both assay methods were based on the assumption that secondary metabolites involved in pathogenicity would be produced in shake culture. Neither assay detected any secondary metabolites toxic to wheat cells. This can either be attributed to a lack of the appropriate stimulus or host factors in the culture or, most likely, because secondary metabolites such as toxins are not involved in pathogenicity in this fungus. Many fungal toxins are produced when a fungus is grown in a general growth medium. An example of this is Cercosporin from *C. oryzae* which is produced when the fungus is grown in Czapek-Dox (Batchvarova *et al.,* 1992), although in some cases an activator for toxin production is required such as a sugar-cane leaf extract to induce HS toxin from *H. sacchari* (Larkin and Sowcroft, 1981). To detect secondary metabolites from *P. herpotrichoides* both a general growth medium and a medium containing wheat cell wall extracts were used, but neither of which stimulated the production of any toxic substances. Thus the necrotic cells of the lesion are probably part of the hosts response to infection and may be elicited by signals from other infected host cells. The detection of cell wall degrading enzymes which have been suggested to be secreted by *P. herpotrichoides* (Hanssler *et al.,* 1971) and their involvement in pathogenicity should be re-investigated using specific methods which would include the growth of the strains and hybrids on pectin which would stimulate and enhance the production of the cell wall degrading pectinases. The lack of enzymes in the non-

pathogenic strains and hybrids would help to clarify their involvement in pathogenicity.

The second part of this work was a comparative time course study on wheat and rye. The object was to relate the appearance of disease symptoms to the presence and abundance of microscopic infection structures and also to an estimate of fungal biomass determined by quantification of the fungal DNA present in the stem bases. This analysis revealed different infection strategies of the W and R types on wheat and rye, the W-type being 'slow and steady' and the R-type being 'fast and furious' in their colonisation of both hosts. Daniels *et al.,* (1991) originally identified the different infection structures of the W and R-types on wheat. Similar infection structures were identified in the current study which expanded on their work by including rye as an alternative host and a time course of infection over 8 weeks. Examination of more W and R-type strains would confirm the infection strategies of 'fast and furious' by the R-type and 'slow and steady' by the W-type. Only single W and R-type strains were examined due to the in-depth studies carried out, although several hybrids were also included in this study, Daniels *et al,* (1991) also only examined a few strains in their study (4 W-types and 4-R-types). Further studies examining these strategies *in vivo* in the presence of other stem base pathogens such as *R. cerealis*, *Fusarium* spp. and *M. nivale* would give a more complete picture of *P. herpotrichoides* infection in the field. The current work suggests that the success of the W or R-type strains appears to relate to the host plant perhaps in its structure or its response to invasion and indicates that the W-type is more adapted to infecting wheat and not rye, the R-types being able to infect both wheat and rye. The success of the R-type on rye may be due to it being able to colonise the leaf tissues before the host defence response is activated or the host is unable to contain it. However because the W-type is slower in its infection it is suggested that the host defence mechanisms can contain the fungus. This idea could be taken forward by examination of the host defence mechanisms such as papillae formation and the hypersensitive reaction in relation to the time in which they occur after initial

invasion by the different pathotypes on both wheat and rye. Previous reports have related temperature to the ability of the W and R-types to infect wheat and rye (Bateman *et al.,* 1990; Higgins and Fitt, 1985a; Hollins *et al.,* 1985). The effect of temperature could also be examined in relation to the host defence mechanisms when under infection by the W and R-types.

The microscopic infection structures of mycelial plates, infection plaques and runner hyphae were all found in advance of the disease symptoms. More infection plaques were produced by the R-type than the W-type which agrees with the findings of the *in vitro* production of infection plaques where the R-types formed a higher number of infection plaques than the W-types. The hybrids that were of low pathogenicity lacked these infection structures indicating the importance of them in successful host colonisation. Infection plaques produced by *P. herpotrichoides* are thought to play a similar role to that of appressoria as seen in *M. grisea.* Daniels *et al,* (1991) demonstrated that the multiple penetration sites found beneath W-type plaques allowed the fungus to penetrate through the leaf sheaths.

Infection plaque 'cells' produced by *P. herpotrichoides* are seen to become pigmented (Daniels *et al.,* 1991; Deacon, 1973) and, as they have a similar function to that of appressorium in penetration on the host cells, an investigation was made as to determine the role of melanin in this fungus. The production of colour mutants which included albinos allowed for an investigation to be made of the involvement of melanin in pathogenicity. The colour mutants were less pathogenic than the wild-types from which they came but were not non-pathogenic unlike the albino mutants from the rice blast pathogen *M. grisea* (Chumley and Valent, 1990). The function of this pigment in *P. herpotrichoides* is environmental protection of hyphae from extremes such as UV irradiation, desiccation and oxidation, thus increasing hyphae longevity and fitness. An oxidative environment is often found as part of the host plant cells in response to invasion. An additional role of melanin is in providing resistance to microbial attack, either protecting substrates from or directly combining

with enzymes such as chitinase and β-1,3-glucanase and. β-1,6-glucanase. Other fungi found at the stem base such as *R. cerealis*, *Fusarium* spp. and *M. nivale* and bacteria may compete with *P. herpotrichoides* and produce some of these anti-microbial compounds. Investigations *in vitro* and *in vivo* on the ability of the colour mutants and wild-types to survive these enzymes would confirm this anti-microbial role of melanin. In *P. herpotrichoides* the protection provided by melanin may be afforded in the hyphae, infection plaques on the host plant and in the pigmented apothecia.

Genetic analysis of the colour mutants with the wild-types suggests that a single gene is responsible for the albino phenotype but the other colour phenotypes appear to have a more complicated genetic basis. An epistasis relationship appears to be involved in the genes for melanin production which is consistent with the order of function, this being albino→ buff → brown/black in melanin biosynthesis. Future investigations detecting the genes involved in its production and their regulation will allow a complete picture to be made of melanin production in this fungus. Further sexual crosses within the colour mutant classes would also further characterise the genetic background of melanin biosynthesis.

The pathway of melanin biosynthesis was found to be via DHN and was determined using the fungicides tricyclazole, fthalide and pyroquilon which are used to control rice blast disease where melanin has been found to be a pathogenicity determinant (Chumley and Valent, 1990). Application of these fungicides in the field which alter melanin biosynthesis could change the ability of the fungal structures such as infection plaques, hyphae and apothecia of *P. herpotrichoides* to survive and thus may reduce the incidence of disease.

The recent development permitting quantification of pathotype specific DNA of *P. herpotrichoides* direct from stem bases and relating this to disease symptoms and microscopic infection structures has allowed progress to be made on understanding

the biology of the pathotypes. Future work may be able to relate the disease symptoms or amount of fungus present to the severity of epidemics and yield loss. Understanding the different infection strategies of the W and R-types may also be applied to the control measures employed, as they are known to respond differently to certain fungicides (Birchmore, Russell and Buschhaus, 1990). The determination of factors crucial in pathogenicity or in survival of this fungus are also important. These combined with understanding host colonisation will help to understand the mechanisms of this pathogen which may also be used in decisions on control methods and their development such as improved cultivar resistance and alternative modes of action of fungicides. These future studies would increase the understanding of this disease and help maintain efficient control into the next century.

CHAPTER 8

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APPENDICES

Appendix 4.1

1 Kb Ladder

1 Kb Ladder	100µl
SDW	300µl
Bromophenol loading dye	50µl

TE Buffer	g l^{-1}
Tris HCl	12.1
EDTA	3.72

Adjusted to pH 8.0, autoclaved

X50 Reaction Buffer

SDW	200mls
Tris HCl	12.11g adjust to pH 8.3
KCl	37.28g
$MgCl_2$	6.1g
Gelatin	0.5%

Gelatin 0.1g ml^{-1} in a beaker of hot water, heat on low power in a microwave for 3 min. Add 1ml per 200mls X50 Reaction Buffer.

Filter sterilised and stored at -20°C

Cresol Red

Cresol Red stock	4mg ml^{-1} in SDW

To use:

Cresol Red stock	1ml
SDW	4ml
Glycerol	5ml

Bromophenol Loading Dye

Bromophenol blue	0.1g
Glycerol	10ml
EDTA	0.372g

Made up to 20ml with 1 X TAE buffer

Ethidium Bromide

Ethidium Bromide	25µg ml^{-1}

CTAB	g l^{-1}
Sorbitol	25.0
Sakosyl	10.0
CTAB	8.0
NaCl	87.65
EDTA	8.0
PVPP	10.0

Made with autoclaved water

X50 TAE Buffer

Tris (base)	242g dissolved in 500ml distilled water
EDTA (disodium)	18.6g
Glacial acetic acid	57.1ml

Made up to 1L, adjusted to pH8.0

Diluted 100mls in 5L distilled water to use

Primer Sequences shown 5'→3'

ITS4	TCCTCCGCTTATTATTGATATGC
ITS5	GGAAGTAAAAGTCGTAACAAGG

TaO5F	GTATCGGACGGAGATCCAGC
TaO5R	TTGCTCAGTGCATTGTCGG
Ty16F	GCGCTGGAAAAAGAGGACTG
Ty16R	TGGAAGGGTCTTGCAGGG

Appendix 4.2

Infection plaque production by strains 23-2 and 22-119 on various surfaces placed on different strengths of MYG.

Surface	MYG Strength	nfection Plaques Present
MYG agar in Petri-dish	Full	Hyphal growth
Scratched MYG agar using a sterile cocktail stick	Full	Hyphal growth
Filter paper placed flat on MYG agar	Full	No growth
Cheese cloth placed flat on MYG agar	Full	yphal growth around cloth
A glass slide placed flat on MYG agar	Full	Few on slide
A scratched glass slide using a sterile scalpel placed flat on MYG agar	Full	Few on slide
Sloped MYG agar in a glass Petri-dish	Full	Many on glass
Slope MYG agar in a plastic Petri-dish	Full	Hyphal growth
Broken plastic placed flat on MYG agar	Full	Hyphal growth
Cellophane placed flat on MYG agar	Full	Hyphal growth
A plastic cover-slip placed flat on MYG agar	Full	Hyphal growth
A glass slide placed flat on MYG agar	Half	Few on slide
A scratched glass slide using a sterile scalpel placed flat on MYG agar	Half	Few on slide
Sloped MYG agar in a glass Petri-dish	Half	Many on glass
Slope MYG agar in a plastic Petri-dish	Half	Hyphal growth
Broken plastic placed flat on MYG agar	Half	Hyphal growth
Cellophane placed flat on MYG agar	Half	Hyphal growth
A plastic cover-slip placed flat on MYG agar	Half	Hyphal growth

Hyphal growth was reduced on half strength MYG compared to full strength MYG.

Appendix 6.1

Time course of UV irradiation applied at 12 J m^{-2} s^{-1} to the colour mutants MC87A (buff) and MC87AA (albino).

UV Exposure Time (min)	MC87A	MC87AA
0	+	+
4	+	+
5	+	+
6	+	+
7	+	+
8	+	+
9	+	+
10	+	+
11	+	+
12	+	+
13	+	+
14	+	+
15	+	+
16	+	-
17	+	-
18	+	+
19	+	+
20	+	-
21	+	-
25	+	+
30	+	-
35	+	-
40	+	-
45	+	-
50	+	-
55	+	-
60	+	-
75	+	-
90	+	-
105	+	-
120	+	-
135	+	-
140	+	-
150	+	-

\+ Re-growth present after 72 hours

\- No re-growth present after 72 hours

Appendix 6.2

Time course of the rate of water loss from colour mutants MC87J (green) and MC87A (buff) desiccated with silica gel.

Desiccation Time (min)	WEIGHT (g)		
	MC87J	MC87A	Cellophane
0	0.6209	0.233	0.1576
30	0.508	0.1473	0.0878
60	0.4209	0.109	0.0842
90	0.3312	0.0998	0.084
120	0.2628	0.0945	0.084
150	0.197	0.0941	0.0935
180	0.154	0.0949	0.084
210	0.137	0.0959	0.084
240	0.133	0.094	0.084
270	0.131	0.093	0.084
300	0.133	0.094	0.084
330	0.132	0.094	0.084
360	0.13	0.092	0.083
390	0.13	0.092	0.084
420	0.03	0.093	0.083
1440	0.0127	0.093	0.0824

Time course of the rate of water loss from colour mutants C87/631/1 (black) and MT178 (albino) desiccated with silica gel.

Desiccation Time (min)	WEIGHT (g)		
	C87/631/1	MT178	Cellophane
0	0.1163	0.119	0.043
30	0.0585	0.0642	0.0304
60	0.048	0.0409	0.029
90	0.047	0.0376	0.0285
120	0.0482	0.0381	0.0293
180	0.0486	0.0384	0.03
240	0.0485	0.038	0.029
300	0.048	0.038	0.0299
360	0.0487	0.039	0.029
420	0.0487	0.0	0.0